UNDERSTANDING THE ROLES OF TRANSCRIPTIONAL REGULATORS FOR THE DEVELOPMENT OF NATURAL AND NOVEL INHIBITORS OF *LISTERIA MONOCYTOGENES*.

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UNDERSTANDING THE ROLE OF TRANSCRIPTIONAL REGULATORS FOR THE DEVELOPMENT OF NATURAL AND NOVEL INHIBITORS OF *LISTERIA MONOCYTOGENES*

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Listeria monocytogenes is pathogenic intracellular foodborne bacterium that causes listeriosis, a rare, but serious disease in humans. Despite the use of antibiotics, the mortality rate remains at 20-30%. The ability of *L. monocytogenes* to survive transmission through food systems and to cause disease is attributed in part to the regulatory networks that control environmental stress adaptation and virulence functions. Therefore, a comprehensive understanding of the factors that are important to virulence, stress response and antimicrobial resistance will help us better develop novel inhibitors for therapeutics. With the information garnered from select regulators, it is possible to identify new drug targets and new drugs for treatment alternatives.

The purpose of this research in *L. monocytogenes* is to (i) determine the contributions of select transcriptional regulators to virulence functions, (ii) assess the contributions of two regulators to antimicrobial peptides response, and (iii) identify novel small molecule inhibitors of the regulator σ^B. In summary, we found that of central transcriptional regulators, σ^B, PrfA, HrcA, CtsR, σ^L, σ^H, and σ^C, σ^B contributes to invasion, PrfA contributes to cell-to-cell growth and CtsR, in addition to PrfA and σ^B, contributes to virulence in a guinea pig model of listeriosis. We determined that σ^B and σ^L are important to controlling expression of genes needed for resistance to the select antimicrobial peptides SdpC and Nisin, thus indicating that σ^B has a role in

virulence and stress survival as well as antimicrobial resistance. Therefore, we focused on σ^B as a promising novel drug target for the treatment of listeriosis. From a library of 57,000 small molecules, we identified a novel compound, sigmastatin, which inhibits the activity of σ^B and its regulon, inhibits *Bacillus subtilis* σ^B and severely impedes *L. monocytogenes* enterocyte invasion. With a solid understanding of the contributions and roles of various regulators in *L. monocytogenes*, novel inhibitors can be used to target those regulators, like σ^B, which are associated with survival, pathogenesis, and resistance. These novel agents can be used to treat listeriosis, extrapolated for use against other similar clinically relevant diseases, or used to gain insight into physiology of pathogenic bacteria and related gene regulation.

BIOGRAPHICAL SKETCH

Mary Elizabeth (Liz) Palmer was born in Elmira, NY in December of 1981 to Mary and William R. Palmer. She attended the University of Virginia, Charlottesville, VA in 2000. There she discovered her passion for benchwork and scientific research while working for Dr. Tyvin Rich on circadian modulation of tumor produced growth factors in the radiation oncology department of the University of Virginia Hospital. After graduation in 2004 with a Bachelor's of Science in Biological Sciences, she worked for a year at the Center for Infectious Diseases and Molecular Medicine at the State University of New York at Stony Brook. Here she dabbled in molecular microbiology, studying pathogens such as *Yesinia pseudotuberculosis* and *Helicobacter pylori*. With a love of pathogens and an interest in studying food safety she headed back to upstate NY (and its bitter winters) to work with Dr. Kathryn J. Boor in the Food Safety Lab in Food Science Department at Cornell University. To the delight of her 98 year old grandmother, she is a third generation Cornellian.

This is dedicated to my family, Dev and Kara.

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LIST OF ABBREVIATIONS

Abbreviation	Abbreviated words
RNAP	RNA Polymerase
CFU	Colony forming unit
P.I.	Post-infection
LAB	Lactic acid bacteria
ECF	Extracytoplasmic function sigma factor
ZOI	Zone of inhibition
SM	Small molecule
SMM	Small-molecule microarray
HTS	High-throughput Screen
DOS	Diversity Oriented Synthesis

LIST OF SYMBOLS

Abbreviation	
σ	Sigma factor
Δ	Delta or delete, usually referring to a genetic deletion

CHAPTER 1

INTRODUCTION

Listeria monocytogenes and human listeriosis

The foodborne pathogen *L. monocytogenes* is an environmentally ubiquitous organism that can easily contaminate processing environments and, thus, food systems (32). While the disease caused by *L. monocytogenes*, called listeriosis, is rare, it can be fatal for susceptible individuals. *L. monocytogenes* infection is typically associated with underlying conditions. Specifically, it poses a serious threat to vulnerable populations, including pregnant women and their fetuses, neonates, the elderly and those with impaired immune systems (13). Among foodborne pathogens, it has one of the highest mortality rates (20%) and accounts for 10% of all foodborne deaths in the U.S. (29). It is capable of breaching three critical barriers intended to protect a human from pathogens: the gastrointestinal barrier, the blood-brain barrier, and the fetoplacental barrier. As a result, it can cause gastroenteritis, septicemia, meningitis, encephalitis, and abortion or stillbirth of a fetus.

Currently, antibiotics are employed for use against *L. monocytogenes* infection (42); however, because of the high case mortality rate caused by listeriosis, improved treatment is needed. Furthermore, various studies have identified single and multidrug resistant strains of *L. monocytogenes* isolated from foods and the environment (18). In one study, 10.9% of *Listeria spp.* and more specifically, 0.6% of *L. monocytogenes* isolated from retail foods displayed resistance to one or more antibiotics (46). In another study, 20 of 21 *L. monocytogenes* strains isolated from cabbage, environmental and water samples were resistant to two or more antibiotics (33). This increasing incidence of multidrug resistant pathogens (17) elicits the concern for lack of antibiotic recourse against the pathogenic bacteria. Thus, attempts to better

characterize the factors that contribute to virulence and antimicrobial resistance in *L. monocytogenes* will help provide a comprehensive understanding of the potential various factors have as viable targets for chemotherapeutic development.

Transcription factors

Transcription factors promote differential expression of various genes depending upon the situational and temporal requirements of the bacterial cell (5). Pathogenic microorganisms require the ability to utilize a variety of proteins to adapt to stressful external conditions both inside and outside the host (47). One method to counter these rapidly changing environments involves employing a myriad of factors that control transcription in a complex multilayered network (2); these factors include alternative sigma factors as well as other activators (such as virulence regulators) and/or repressors (such as heat shock regulators) (21).

Alternative sigma factors dissociably interact with core RNA polymerase (RNAP), recognize certain promoter sequences and direct transcription of target gene sets critical to combating stress (21), resisting antimicrobials (3, 31) and maintaining viability and sustaining infection in the host (11, 30, 36). *L. monocytogenes*, for example, is exposed to adverse conditions in the environment, in food systems, during transmission and in the mammalian host. Therefore, in addition to general house keeping sigma factor σ^A, *L. monocytogenes* utilizes 4 alternative sigma factors, σ^B, σ^C, σ^H and σ^L to survive exposure. General stress response sigma factor σ^B in particular aids *L. monocytogenes* in withstanding external stressors including acidic pH encountered in the stomach (pH 2), bile salts in the duodenum, and elevated osmolarity throughout the intestinal tract (8, 14, 15, 35). In addition to conferring protection to adverse environmental elements, which can be a prerequisite to virulence (44), σ^B has also been shown to play a role in pathogenicity of *L. monocytogenes*. σ^B is important to regulating the transcription of a number of virulence genes including

inlAB (encoding two genes critical to attachment and invasion (26, 27)), *bsh* (encoding bile salt hydrolase needed for gastrointestinal passage (10, 12)) and *prfA* (encoding PrfA, which is the global virulence gene regulator critical to infection (45)) through upstream σ^B-dependent promoters (25). By regulating genes such as these, σ^B contributes to the establishment of infection in mammals (7, 19, 43). σ^B has also been shown to contribute to virulence in other gram positive human pathogens, including *Bacillus anthracis* (16) and *Staphylococcus aureus* (24, 28). While the other alternative sigma factors in *L. monocytogenes* are important for surviving select stresses, it is well established that σ^B has a prominent role in stress resistance and virulence, making it an excellent target of focus for developing and identifying novel anti-infective compounds.

Targeting L. monocytogenes using chemical biology

Utilizing high-throughput chemical biology platforms for pharmaceutical development, researchers have the ability to extend drug discovery to identify novel small-molecule therapeutics from millions of compounds (1). Small molecules are simple organic chemical compounds, typically of low molecular weight, which can have useful biological effects (39). They are vital to biological functioning of living organisms (e.g. small molecules can function as hormones or neurotransmitters) and can bind macromolecules such as DNA, RNA or proteins to alter their activity (37, 40). Small molecules are frequently used for medicinal purposes (37) and have been for centuries (i.e. plant and fungal extracts). The first isolated natural small molecule was morphine, which was derived from an opium plant and subsequently sold for medicinal purposes by Heinrich Emanuel Merck (23). In addition to naturally occurring products made by living cells (9), chemists also create synthetic small molecules by combining chemical building blocks, such as ethanol or benzene (6). Using a combinatorial synthesis approach, chemists can realize vast combinations of

core functional groups to create complex and diverse small molecules (4). This process of synthesizing combinatorial libraries of diverse compounds is called diversity-oriented synthesis (DOS) (41). Many of these synthetic compounds are modeled after naturally-occurring bio-active small molecules (41) because natural compounds have proven to be very effective for use in treatment.

With their ability to cause phenotypic changes and modulate cell functions (37), small molecules can be used as probes for understanding biological systems (40), which then aids in the development of potential therapeutic drugs. This field of study is called chemical biology or chemical genetics because it employs chemical compounds to study genetics. For example, in the same way classical geneticists create gene mutations to alter the function of a single-gene product, chemical biologists use exogenous small molecules to alter the function of a single-gene product (39). Both approaches provide a more complete understanding of the biological consequences within a cellular context. When small-molecule screens are performed in a high-throughput format, chemical biologists expand current knowledge about biological processes and phenotypic consequences to identify novel and medicinally-hopeful perturbational agents (perturbagens) in a rapid and comprehensive manner. This approach can effectively be used to identify novel small molecules for targeting diseases caused by prokaryotes. The extensively studied intracellular pathogen, *L. monocytogenes*, is an ideal model for identifying small molecule agents for treating bacterial infections. By selecting specific biological targets in *L. monocytogenes* that are common to Gram-positive pathogens, knowledge garnered pertaining to drug discovery can be extrapolated for other bacteria. Also, attenuating the pathogen's virulence and stress response attributes without killing it can eliminate selective pressure caused by disruption of essential gene functions (as done by classical antibiotics). Alleviating this pressure makes the pathogen susceptible to

pharmacological inhibitors ideally without eliciting resistance, reducing the likelihood of developing more bacteria impervious to the effects of antibiotics (34).

Utilizing this approach, ground-breaking work performed by Hung et al. in *Vibrio cholerae*, demonstrated that small molecules can inhibit essential molecular processes required for transcription of virulence genes in *V. cholerae* (22, 38). Based on that research, we hypothesize that certain synthesized and/or naturally-derived small molecules targeting a specific biological factor, such as a transcription factor, will hinder the infective process of *L. monocytogenes*. A target of particular interest in *L. monocytogenes* is general stress response sigma factor B, σ^B. As previously mentioned, it is an ideal target for inhibition by small molecules because it is important to both virulence and stress response and it is common to several significant human pathogens, such as those in the genera *Bacillus* and *Staphyloccocus* (44). Further impetus for targeting a factor, such as σ^B, which is specific to certain bacteria, is the aim of identifying well tolerated chemotherapy agents, which are not harmful to the mammalian host (20).

Therefore, focusing our understanding on transcriptional regulators (and their interactions), which contribute to the regulation of stress survival, antimicrobial resistance and virulence gene repertoire of *L. monocytogenes*, will ultimately provide a solid information base, which we can use to develop novel inhibitors of factors critical to pathogenesis. This will improve the search for new efficacious anti-infective drug candidates against pathogenic organisms, such as *L. monocytogenes* and may boost our understanding of the finer aspects of gene-regulation.

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CHAPTER 2

CONTRIBUTIONS OF MULTIPLE TRANSCRIPTIONAL REGULATORS TO *LISTERIA MONOCYTOGENES* VIRULENCE FUNCTIONS

ABSTRACT

The foodborne pathogenic bacterium, *Listeria monocytogenes*, causes listeriosis, a rare, but serious, invasive disease affecting both humans and animals. The ability of *L. monocytogenes* to survive transmission through food systems and to cause disease is attributed to both its environmental stress survival capabilities and its virulence gene repertoire. Stress response and virulence functions in *L. monocytogenes* have been ascribed to the pleiotropic transcriptional regulators σ^B and PrfA, however, little is known about the involvement of other transcriptional regulators in pathogenicity. To assess contributions of various regulatory proteins to virulence and virulence-associated phenotypes, *L. monocytogenes* laboratory parent strain 10403S and a collection of otherwise isogenic strains each bearing in-frame deletions in genes encoding alternative sigma factors σ^L, σ^H, or σ^C or repressors CtsR or HrcA were tested in: (i) invasion assays with Caco-2 intestinal epithelial cells; (ii) intracellular growth assays in J774 mouse macrophage-like cells; and (iii) intragastric infections in the guinea pig model. We found that σ^B was essential for optimal invasion efficiency in intestinal epithelial cells and only PrfA was obligatory for wildtype cytosolic growth and spread. In addition to PrfA and σ^B, we found that CtsR also contributes to virulence during intragastric infection in the guinea pig. In summary, while no clear virulence-associated phenotypes were attributed to *L. monocytogenes* σ^L, σ^H, σ^C, or HrcA under the conditions tested, it is possible that regulatory networks exist among these regulators to enable at least partial functional compensation in the absence of a given factor.

INTRODUCTION

Rapid adaptation to stress and the external environment affords the intracellular pathogen *Listeria monocytogenes* the ability to survive and persist in various niches, including mammalian hosts (6, 9). As a result *L. monocytogenes* causes a severe invasive disease, listeriosis, with a 20-30% fatality rate in the US (22). A network of regulatory proteins, such as alternative sigma factors (σ^B, σ^L, σ^H, σ^C), two-component regulators, and other transcriptional activators or repressors (i.e. PrfA, CtsR, HrcA), guides RNA polymerase to recognize certain promoter sequences. Utilization of this network permits coordination of signals and elicits responses by promoting differential expression of specific genes. The complex concerted effort of these regulatory proteins allows *L. monocytogenes* to sense and respond with specificity and fine tuning that allows survival and infection. Therefore, investigation into mechanisms through which *L. monocytogenes* controls virulence may provide insight and guidance for development of more effective disease intervention strategies.

Virulence roles for the global virulence regulator PrfA and the general stress sigma factor σ^B have been characterized and data indicate that PrfA and σ^B work in concert to coordinate the infectious process (3, 25, 31, 32). Virulence roles for others transcription factors, including alternative sigma factors σ^L, σ^H, σ^C and class I and III stress response repressors HrcA and CtsR, respectively, have been less extensively studied. While σ^L has been shown to contribute to carbohydrate metabolism and antimicrobial resistance (1, 26, 29), σ^H is important for growth in minimal or alkaline media (28) and σ^C, a *L. monocytogenes* lineage II-specific extracytoplasmic function (ECF) sigma factor, is important for response to heat stress (33). Currently, these alternative sigma factors have not been indicated in virulence regulation. However, CtsR and HrcA, which negatively regulate genes important for survival of stress, including heat shock (12, 23, 24), control genes upregulated *in vivo* and encode

proteins relevant to virulence (3), such as proteases and chaperonins. The repressor CtsR, regulates virulence-associated Clp proteases. Under stress conditions, CtsR repression is relieved and Clp proteases degrade damaged proteins, allowing the cell to tolerate stress (4) and promote escape from phagosomes (30). Furthermore, HrcA binds operators and represses transcription of genes encoding chaperonins, such as DnaK, which is up regulated intracellularly in macrophages (12) and GroE, which helps the bacterium survive phagocytosis (8) and the vacuolar compartment (4). Moreover, transcriptomic analysis of CtsR and HrcA in the *L. monocytogenes* strain 10403S (2), showed that CtsR and HrcA regulate genes encoding proteins important to acid and metabolic stress as well as virulence (14). Many of these genes are co-regulated by σ^B, including *hrcA* itself (13) suggesting multiple layers of regulatory control amongst these regulatory proteins.

To build a more comprehensive understanding of the role each regulator plays in virulence-associated functions, we performed *in vitro* assays, using single and double in-frame deletions of the regulators. We know that σ^B is essential for attachment and infection of enterocytes (in Caco-2 human intestinal epithelial cells (17)) and that PrfA is important for *L. monocytogenes* replication in macrophages (7). It was also shown that, in addition to PrfA, σ^B is required for a successful *L. monocytogenes* gastrointestinal infection in guinea pigs (11). Therefore, with this knowledge we evaluated σ^B, σ^L, σ^H, σ^C, CtsR, HrcA and PrfA to pinpoint and/or reaffirm the contributions of each protein to invasion, intracellular growth, and *in vivo* infection. We also began assessing the interactions between select regulators. Phenotypic characterization of the regulators during critical aspects of the infectious cycle provides a foundation for understanding the contributions of each protein in addition to working towards unraveling and understanding the complex transcriptional regulatory networks coordinated by these proteins.

MATERIALS AND METHODS

Bacterial strains

L. monocytogenes 10403S and eleven single and double isogenic mutant strains, Δ*sigB*(31), Δ*sigC* (Chaturongakul, unpub.), Δ*sigH* (Chaturongakul, unpub.), Δ*sigL*(26), Δ*ctsR* (14), Δ*hrcA* (13), Δ*prfA* (32), Δ*sigB/*Δ*sigH* (Chaturongakul, unpub.), Δ*sigB/*Δ*ctsR* (14), Δ*ctsR/*Δ*hrcA* (13), and Δ*sigB/*Δ*hrcA* (13) previously created in *L. monocytogenes* 10403S using splicing-by-overlap extension (SOE) PCR and allelic exchange mutagenesis used in this study (Table 2.1). Select single mutants including Δ*sigC*, Δ*sigH*, Δ*sigL*, Δ*ctsR*, Δ*hrcA* were also assessed in the animal model. Strains were grown to stationary phase, i.e., OD_{600}=0.8 +1hr, allowing comparison to previous assessments of regulators sigB and PrfA using cells grown to stationary phase (11).

Invasion assay

The human colorectal adenocarcinoma epithelial cell line Caco-2 (ATCC HTB-37) was cultured and invasion assays were performed as described by Garner et al. 2006 (11) with minor modifications. Briefly, 5.0 x 10^4 Caco-2 cells were seeded into 24-well plates (Costar, Corning, NY) 48 h prior to infection. For infection, the Caco-2 cells were inoculated with approximately 2.0x 10^7 *L. monocytogenes* cells (grown to stationary phase, i.e., OD_{600}=0.8 +1hr); exact *L. monocytogenes* numbers used for infection were determined by plating on BHI agar. Intracellular *L. monocytogenes* numbers were determined 90 min post infection as previously described (11). Invasion efficiency was calculated as the number of bacteria recovered relative the number of bacteria used for inoculation (i.e., log (CFU/ml recovered/ CFU/ml inoculated). Data represent 4 independent experiments. Data were analyzed using one-way analysis of variance (ANOVA) and Dunnett's t-test, performed in SAS® 9.0 (SAS Institute). Averages for three of four replicates for

Table 2.1: Strains used in this study

Strain	Genotype	Reference
L. monocytogenes FSL X1-001	parent strain 10403S	Bishop and Hinrichs, 1987 (2)
L. monocytogenes FSL A1-254	10403S Δ*sigB*	Wiedmann et al., 1998 (31)
L. monocytogenes FSL C3-126	10403S Δ*sigH*	Chaturongakul, unpublished
L. monocytogenes FSL B2-124	10403S Δ*sigL*	Chaturongakul, unpublished
L. monocytogenes FSL C3-113	10403S Δ*sigC*	Chaturongakul, unpublished
L. monocytogenes FSL B2-046	10403S Δ*prfA*	Wong et al., 2004 (32)
L. monocytogenes FSL H6-190	10403S Δ*ctsR*	Hu et al., 2007 (14)
L. monocytogenes FSL B2-101	10403S Δ*hrcA*	Hu et al., 2007 (13)
L. monocytogenes FSL C3-123	10403S Δ*sigB*/Δ*sigH*	Chaturongakul, unpublished
L. monocytogenes FSL H6-193	10403S Δ*sigB*/Δ*ctsR*	Hu et al., 2007 (14)
L. monocytogenes FSL H6-194	10403S Δ*sigB*/Δ*hrcA*	Hu et al., 2007 (13)
L. monocytogenes FSL H6-198	10403S Δ*ctsR*/Δ*hrcA*	Hu et al., 2007 (13)

10403S, Δ*sigB,* Δ*ctsR,* Δ*brcA,* Δ*sigB/*Δ*ctsR,* Δ*sigB/*Δ*hrcA,* Δ*ctsR/*Δ*hrcA* were represented previously (13). To determine if there were statistically significant interaction effects between the *sigB* and the *sigH, sigB* and the *ctsR, sigB* and the *hrcA,* and the *ctsR* and the *hrcA* deletions, a two-way ANOVA (with Dunnett's t-test) was performed. Interaction is a term in a statistics model in which the effect of one variable on an outcome is a function of another variable. In our model, the dependent variable was invasion efficiency; the independent variables included sigB + sigH + sigB*sigH + replicate. The factors "sigB" and "sigH" in the model, for example, indicate the presence or absence of that gene in the strains tested. This model was used to similarly assess the contributions of both genes in the other double mutant strains.

Intracellular growth assay

The mouse macrophage-like cell line J774A.1 (ATCC TIB-67) was cultivated at 37°C with 5% CO_2 in Dulbecco's Modified Eagle Medium (DMEM) with Earle's salts and 1% Sodium Pyruvate (Gibco; Gaithersburg, MD) containing 10% fetal bovine serum (Gibco), 1.5 g/L Sodium Bicarbonate (Gibco), and 100μg/ml each Penicillin G and Streptomycin (J774 medium). At approximately 48 h before intracellular growth assays, J774 cells were seeded at a density of $2x10^5$ cells/ml in each well of a 24 well plate using J774 medium without antibiotics. In order to activate macrophages, J774 cells were shifted to J774 media without antibiotics containing lipopolysaccharide (LPS; Sigma, St. Louis, MO) at final concentration of 100ng/ml 24 hours before infection. At 30 min prior to assay, fresh media without antibiotics was added. J774 cells were then inoculated with *L. monocytogenes* at a multiplicity of infection (MOI) of 1. *L. monocytogenes* used for infection were grown to early stationary phase (defined as OD_{600}=0.8 +1hr) in BHI, flash frozen and stored in liquid nitrogen before the assays; bacterial numbers were determined (on BHI agar

plates) after freezing and immediately prior to the assays. At 30 min post inoculation, the J774 cells were washed with 1ml sterile PBS, followed by addition of 1ml of fresh media with 50μg/ml gentamicin. At 1.5, 3.5, 5.5, and 7.5 hr post-inoculation, inoculated J774 cells in different wells were washed three times with 1ml of sterile PBS and lysed with 500ul of ice cold sterile distilled water, followed by plating of the cell suspension on BHI agar to determine intracellular bacterial numbers at each time point. Intracellular growth was calculated as the number of bacteria recovered at each time point relative to the number of bacteria recovered at t=1.5 (i.e., [log CFU/ml t=x] - [log CFU/ml t=1.5]. Data represent 5 independent experiments. Data were analyzed using one-way ANOVA and Tukey's studentized range (HSD) test, performed in SAS® 9.0 (SAS Institute).

Animal care and housing condition

Animal protocols (# 2002-0060) were approved by the Institutional Animal Care and Use Committee prior to initiation of the experiments. Male Hartley guinea pigs (Elm Hill, Chelmsford, MA) weighing approximately 300g at about 3 weeks of age were housed individually allowing for collection of each animal's fecal material. Animals were provided with feed and water ad libitum. Cages were changed daily, and animal health and weight were monitored and recorded daily. Animals were acclimated for 5 days prior to infection.

Intragastric infection of guinea pigs

Intragastric infections of guinea pigs were performed as described previously (11). Briefly, animals were anesthetized with isoflurane administered via inhalation and *L. monocytogenes* (1×10^{10} CFU) was inoculated intragastrically after stomach pH was buffered using by administrating 1.5 ml of PBS containing 125 mg calcium carbonate (pH 7.4). Data represent 4 independent experiments.

Enumeration of *L. monocytogenes* from organs

Animals were euthanized for organ harvest at 72 h p.i. as was previously established by (20). The liver, mesenteric lymph nodes (MLN) and small intestine (a 20-cm portion, immediately proximal to the cecum) were aseptically removed, and processed according to Garner et al. 2006. Additionally, to confirm *L. monocytogenes* presence for each organ, 10ml of homogenate was added to 90ml of Listeria Enrichment Broth (Becton Dickenson, Sparks, MD) and incubated at 30°C for 48hrs, after which it was streaked onto Oxford agar (Oxoid, Ogdensburg, NY). After incubation at 30°C for 48 h, colonies exhibiting Listeria-like morphology were recorded as *L. monocytogenes*.

Enumeration of *L. monocytogenes* from feces

Fecal pellets from each animal were collected daily and enumerated according to Garner et al., 2006. After incubation at 30°C for 48 h, colonies exhibiting Listeria-like morphology were counted and recorded as *L. monocytogenes*. *L. monocytogenes* identification was confirmed on a representative subset of these colonies by plating on LMPM agar (Biosynth Biochemica & Synthetica, Naperville, Ill.).

Statistical analyses

Data were analyzed using general linear model (GLM) with Tukey's studentized range (HSD) test or Dunnett's t-test. Analysis was performed with Statistical Analysis Software (SAS) 9.0 (SAS Institute, Inc., Cary, NC).

Histopathology and immunohistochemistry

Histopathology and immunohistochemistry was performed by Brad Njaa and interpreted by Brad Njaa and Rachel Peters. Tissues from euthanized guinea pigs were fixed in 10% buffered formalin for a minimum of 48 h. Fixed tissues were processed using a Tissue Tek VIP E 300 (Sakura Finetek U.S.A., Inc. Torrance, CA) in preparation for paraffin embedding in a Tissue Tek embedding station (Sakura Finetek U.S.A., Inc.). Formalin-fixed, paraffin-embedded tissues were sectioned at a thickness

of 6 um, placed on glass slides, and stained with hematoxylin and eosin for microscopic evaluation. *L. monocytogenes* immunohistochemistry was performed as previously described (15) with minor modifications, as follows. Briefly, formalin-fixed, paraffin-embedded tissues were sectioned to a thickness of 6 um and deposited on Probe-On glass slides. For each organ section investigated, one slide was stained using a polyclonal antibody to *L. monocytogenes* (Becton Dickinson, Sparks, MD), while the second slide was stained with a nonspecific antibody. The secondary antibody was an anti-immunoglobulin G antibody. All slides were stained using the avidin-biotin system, and the chromogen was diaminobenzidine. All slides were examined using an Olympus BX41 microscope. Photomicrographs were taken using a Q Imaging micropublisher 5.0 RTV (Burnaby, British Columbia, Canada) and a 50x or 100x oil objective lens.

RESULTS

Caco-2 intestinal epithelial cell assay demonstrates the requirement of σ^B for *L. monocytogenes* attachment and invasion.

To assess the invasion capacity of various transcriptional regulator mutants in Caco-2 human enterocytes, we used laboratory parent strain 10403S and isogenic single mutants of regulators σ^B, σ^L, σ^H, σ^C, CtsR, HrcA and PrfA, as well as, strains containing double deletions of select regulators of interest, such as Δ*sigB*/Δ*sigH*, Δ*sigB*/Δ*ctsR*, Δ*sigB*/Δ*hrcA*, and Δ*ctsR*/Δ*hrcA* (Figure 2.1). We found that in line with previous assessments (10, 17), the Δ*sigB* strain showed significantly reduced invasion capacity ($p<0.05$). Two double mutant strains, Δ*sigB*/Δ*ctsR* and Δ*sigB*/Δ*hrcA* showed significantly lower invasion than 10403S ($p<0.05$). The Δ*sigB*/Δ*hrcA* strain invasion capacity was similar to that of the single *sigB* knockout strain. Two-way ANOVA analyses of invasion data showed no significant "SigB*HrcA" interaction effect on

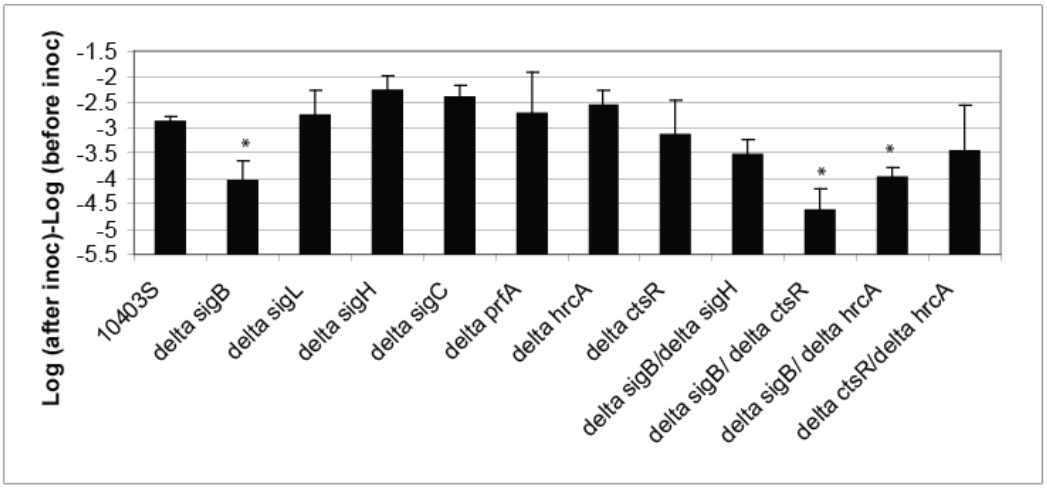


Figure 2.1: Invasion efficiency of *L. monocytogenes* transcriptional regulator mutant strains in the intestinal epithelial cell line Caco-2. Data shown represent the average of four independent experiments. "*" indicates mutant was significantly different ($p<0.05$) than the 10403S parent strain (GLM, Dunnett). Two-way ANOVA showed no interaction effect for double mutants. Portions of this data have been published previously (13), see materials and methods.

invasiveness ($p>0.05$). The Δ*sigB*/Δ*ctsR* strain showed >0.5 log lower invasion than the Δ*sigB* strain. Initial analysis based on partial set of the data indicated an interaction of σ^B and CtsR on invasion (13), however, further analysis of complete set of data using two-way ANOVA analyses of "SigB*CtsR" indicated no significant interaction of σ^B and CtsR on invasion. This could mean that the lower invasion of Δ*sigB*/Δ*ctsR* (lower than either of the respective single mutants) is probably a result of additive (rather than multiplicative) contributions of σ^B and CtsR to invasiveness. Though neither the Δ*ctsR* strain nor the Δ*hrcA* strain exhibited reduced invasiveness, the Δ*ctsR*/Δ*hrcA* strain exhibited lower invasion than either of the single mutants, suggesting that the loss of both proteins produces a compounding effect on invasion capacity. However, statistical analyses of invasion data showed no significant "CtsR*HrcA" statistical interaction effect on invasiveness ($p>0.05$). The Δ*sigH* strain exhibited higher invasion than its parent (though not statistically significant). Interestingly, the effect of deleting *sigH* in the Δ*sigB*/Δ*sigH* strain appeared to have moderated the effect on invasion produced by loss of intact *sigB* gene. Specifically, the Δ*sigB*/Δ*sigH* strain did not exhibit reduced invasion like all other strains with a Δ*sigB* background (Figure 2.1). The statistical interaction effect of "SigB*SigH" was not significant, though ($p>0.05$).

Intracellular growth assay indicates none of the selected regulators, other than PrfA, contribute to intracellular growth and spread.

As *L. monocytogenes* is a facultative intracellular pathogen, we investigated a potential role for the transcriptional regulators in replication and spread in the intracellular microenvironment. In order to determine if the transcriptional regulator mutants were inhibited in their ability to adapt and grow once the bacteria were cytosolic, we performed intracellular growth assays using LPS-activated J774 mouse

Table 2.2. Intracellular growth assay results in J774 cells

Time Post inoculation (hr)	Mean Log((CFU/ml t=x)/(CFU/ml t=1.5hr)) (SD) for *L. monocytogenes* strains[a]											
	10403S	Δ*sigB*	Δ*sigL*	Δ*sigH*	Δ*sigC*	Δ*prfA*	Δ*hrcA*	Δ*ctsR*	Δ*sigB*/ Δ*sigH*	Δ*sigB*/ Δ*ctsR*	Δ*sigB*/ Δ*hrcA*	Δ*ctsR*/Δ*hrcA*
1.5 hrs	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
3.5 hrs	0.76 (0.21)	0.6 (0.21)	0.52 (0.19)	0.68 (0.13)	0.65 (0.17)	-0.14* (0.21)	0.71 (0.20)	0.67 (0.27)	0.70 (0.14)	0.65 (0.16)	0.62 (0.20)	0.61 (0.12)
5.5 hrs	1.27 (0.09)	1.14 (0.18)	1.07 (0.18)	1.16 (0.31)	1.11 (0.32)	-0.30* (0.12)	1.03 (0.47)	1.13 (0.20)	1.13 (0.27)	1.09 (0.28)	1.18 (0.20)	1.11 (0.15)
7.5 hrs	1.75 (0.22)	1.71 (0.10)	1.58 (0.21)	1.90 (0.21)	1.76 (0.07)	-0.21* (0.17)	1.72 (0.13)	1.76 (0.23)	1.67 (0.21)	1.70 (0.20)	1.72 (0.29)	1.73 (0.17)

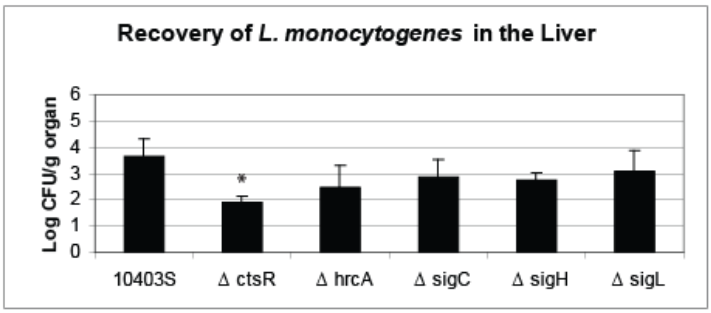
[a] "*" indicates values that are significantly different ($P<0.05$; one way ANOVA with Tukey's studentized range (HSD) test). Data represent 5 independent experiments.

SD indicates standard deviation.

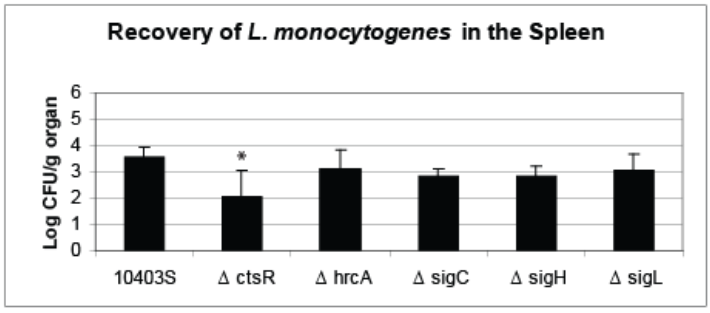
macrophage-like cells. The number of bacteria recovered at t=3.5, 5.5 and 7.5 hours post-infection (p.i.) were normalized to their respective number of bacteria recovered at initial invasion (t=1.5 h p.i.), in order to assess growth after and separate from invasion. This assay showed that σ^B does not directly contribute to intracellular growth in the macrophage, as its respective mutant and double mutants showed no inability to grow as compared to its parent strain. Likewise, σ^L, σ^H, σ^C, CtsR, and HrcA also did not contribute to intracellular growth, as their respective deletion mutants exhibited no hindrance of growth within the macrophages (Table 2.2). As seen previously (7), however, Δ*prfA* showed a complete loss in ability to multiply inside the cell ($p<0.05$; Table 2.2).

Guinea pig model of listeriosis indicates CtsR contributes to *L. monocytogenes* virulence.

We used a guinea pig model of listeriosis to evaluate *in vivo* contributions of transcriptional regulators to virulence. It was previously discovered in our laboratory, that Δ*prfA* strain is avirulent and Δ*sigB* is virulence attenuated, thus they are both required for a successful *L. monocytogenes* gastrointestinal infection in guinea pigs (11). In order to gain a more comprehensive understanding of the roles of the other transcriptional regulators (σ^L, σ^H, σ^C, CtsR and HrcA) in comparison to PrfA and σ^B to virulence *in vivo*, we infected each guinea pig separately with $1x10^{10}$ CFU of each *L. monocytogenes* mutant in our panel of transcriptional regulators. Interestingly, we found that Δ*ctsR* was significantly less virulent than 10403S, as the animal infected with Δ*ctsR* had the lowest recovery (in log CFU/g organ) of *L. monocytogenes* in samples taken from the liver, spleen, mesenteric lymph nodes and the distal section of the ileum ($p<0.05$; Figure 2.2). Δ*ctsR* was recovered from all tissues ~ 1 to 1.5 Log CFU/g less than 10403S. The relative contributions of the other transcriptional



(a)



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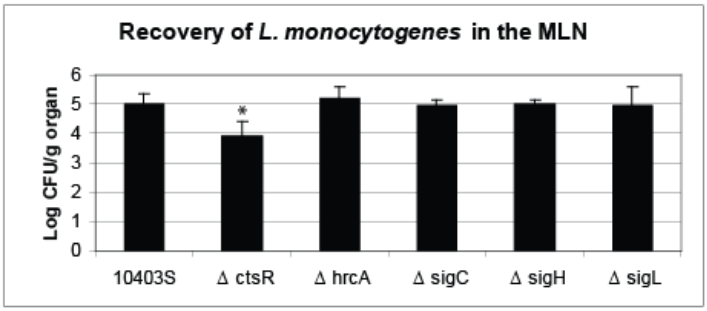

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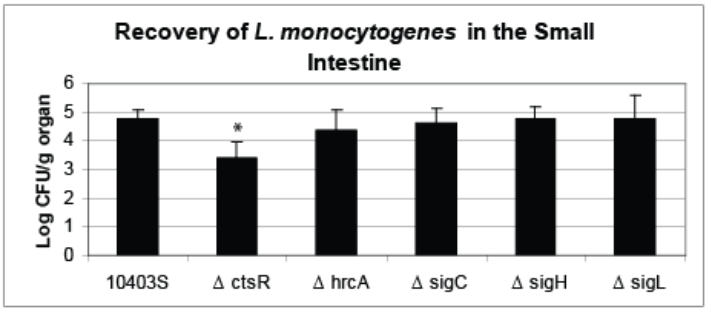


(d)

Figure 2.2: Recovery of *L. monocytogenes* strains from organs of infected guinea pigs. Data shown represent the average of 4 independent experiments. "*" indicates that recovery of a mutant was significantly different ($p<0.05$) from 10403S (GLM, Tukey HSD). MLN indicates mesenteric lymph node.

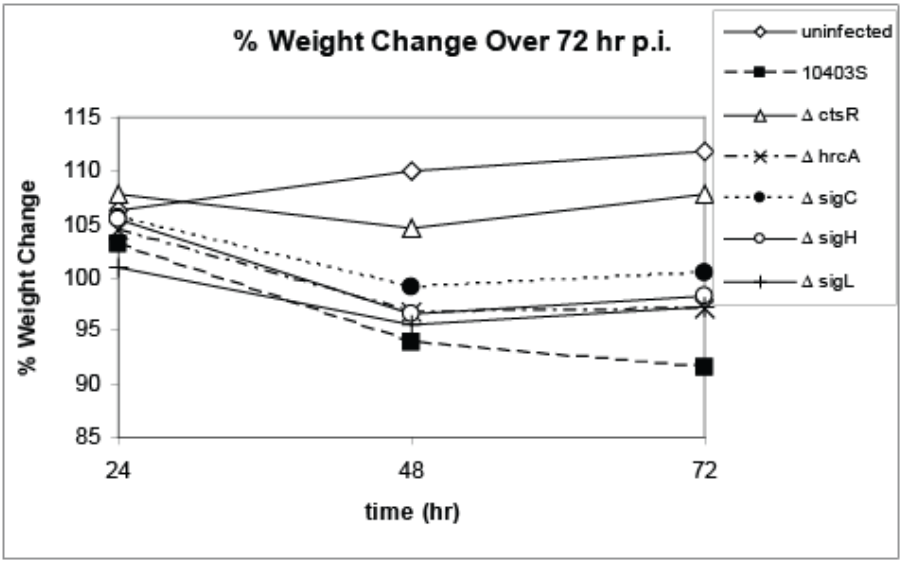
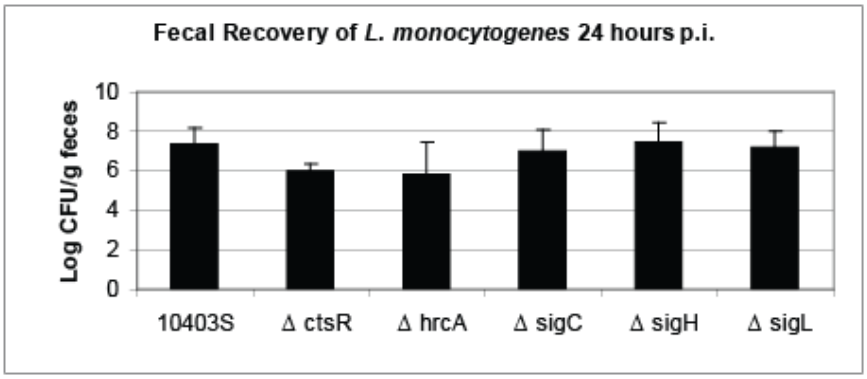
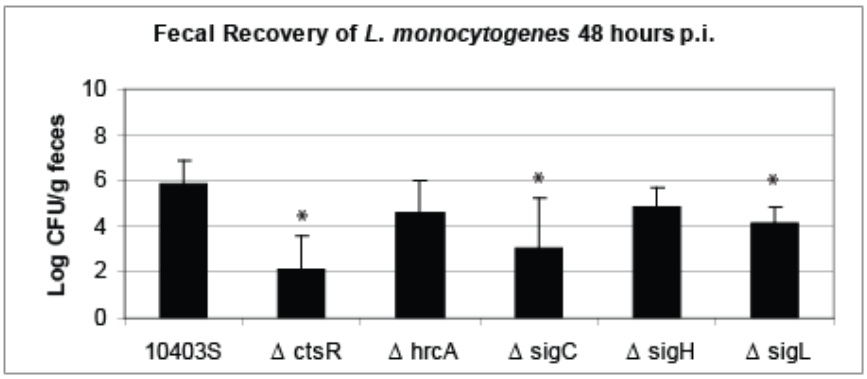


Figure 2.3: Weight development for guinea pigs infected with various *L. monocytogenes* regulator mutants over 72 hours post-infection. Data shown represent the average of four independent experiments. Data were analyzed using general linear model (GLM) with Dunnett's t-test.



(a)



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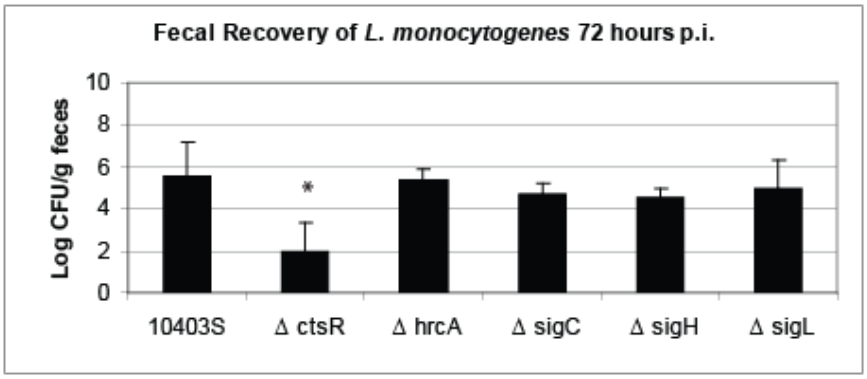

(c)

Figure 2.4: Recovery of *L. monocytogenes* strains from fecal specimens eliminated from infected guinea pigs. Data shown represent the average of four independent experiments. "*" indicates that recovery of a mutant was significantly different ($p<0.05$) than that of 10403S (GLM, Dunnett).

regulators were negligible as their respective mutants showed no detectable differences or attenuation in virulence as compared to 10403S. According to histopathology, differences in tissue samples were minimal among guinea pigs infected with the various strains. The infection proceeded over 72 hours, during which the animal infected with Δ*ctsR* exhibited no physiological signs of distress, whereas the other animals demonstrated some anorexia. In fact, as compared to 10403S, the animals infected with Δ*ctsR* strain had the greatest positive % weight change, approximately 16% higher than 10403S at 72 hrs post-infection ($p<0.05$; Figure 2.3), similar to the uninfected control. This Δ*ctsR*-infected animals' average weight change was similar to that of Δ*sigB*-infected animals in Garner et al.(11). Animals infected with the Δ*ctsR* strain also eliminated the least bacteria in their fecal matter, approximately 4 Log CFU/g less than animals infected with 10403S at both 48 and 72 hours post-infection ($p<0.05$; Figure 2.4). Both animals infected with Δ*sigL* and Δ*sigC* eliminated significantly less bacteria than 10403S at 48 hours post-infection ($p<0.05$), however, not at any other time point.

DISCUSSION

A bacterium's cellular adaptation to external conditions is largely dependent on differential expression of genes through the activity of multiple regulatory proteins including transcription factors. These complex and dynamic interactions between various regulatory proteins are critical to stress response (5) and the establishment of infection in a mammalian host for the intracellular pathogen *L. monocytogenes* (3, 25). Using phenotypic analysis to assess the contributions of seven transcriptional regulators, we determined whether and at what point each protein aids in the coordinated regulation of virulence and virulence-associated functions. Overall our data show that PrfA and σ^B are important to intracellular growth and entry,

respectively (3, 25), and that CtsR is an emerging factor that also promotes a successful infection *in vivo*. Simultaneously, we found that the other regulators examined (σ^L, σ^H, σ^C, and HrcA) do not seem to have apparent roles in virulence based on the conditions assessed here.

Using an *in vitro* model of interaction between *L. monocytogenes* and intestinal cells, we observed that σ^B is indeed necessary for optimal invasion efficiency, as there was an appreciable loss in invasion capacity of Δ*sigB* as compared to its isogenic parent. This is consistent with its known role regulating *inlA* and *inlB* (27), thereby mediating attachment and early invasion in intestinal epithelium and was previously seen (17, 18). Also in agreement with previous work (18), Δ*prfA* grown to early stationary phase at 37ºC, showed no significant impediment in ability to invade as compared to its parent strain, further exemplifying its relatively dispensable role in invasion. We did see that in a Δ*sigB*/Δ*sigH*, the loss of σ^H moderates the lowered invasion capacity as a consequence of the loss of σ^B, resulting in no reduction in invasion. This may simply indicate that σ^H selectively regulates genes to reduce invasiveness and σ^B regulation increases invasiveness. Similar interplay has been seen between PrfA and σ^B, in which σ^B moderates the effects of PrfA activity conferring to the cell better control during infection (25). Previous transcriptional profiling assessments indicate σ^B and negative transcriptional regulators CtsR and HrcA share overlapping transcriptomes (13, 14). This commingling of gene regulation indicates these proteins interact in a coordinated fashion to promote stress survival (13) and possibly virulence. Most notably, while the single mutant of the gene encoding the repressor CtsR did not exhibit loss of invasion capacity, we found that the Δ*sigB*/Δ*ctsR* strain exhibited invasion efficiency lower than a Δ*sigB* strain, consistent with previous reports (13, 14). This may indicate that regulator redundancy between

σ^B and CtsR causes an increased impairment in invasion; functional redundancy has been observed among alternative sigma factors in *Bacillus subtilis* (21).

After examining the roles of the transcriptional regulators in replication and cell-to-cell spread, we found that only PrfA has an evident role in this aspect of *L. monocytogenes* pathogenesis. We surmise that under these conditions in the chosen cell line, the transcriptional regulators are not involved in intracellular growth during the infection process and are likely more important to a niche outside the host. Alternatively, they may be able to compensate for one another such that defects could not be detected in this assay.

It has been shown that the guinea pig model of intragastric infection is the most representative of human listeriosis as transmitted through the ingestion of contaminated foods (11). The guinea pig was chosen for our *in vivo* assessments as it is a naturally permissive host for *L. monocytogenes* infection via the gastrointestinal route (19). While Δ*prfA* is avirulent and Δ*sigB* is virulence attenuated indicating both PrfA and σ^B are required for full scale infection in a guinea pig (11), our *in vivo* assay showed that CtsR is also important for *L. monocytogenes* pathogenesis. This correlates with known functions of CtsR. Surprisingly despite indications that, like CtsR, HrcA regulates genes important to virulence (*dnaK, groESL*) (4, 12), the Δ*hrcA* strain showed no attenuation of virulence in the guinea pig model.

CtsR, encoded by *lmo0229*, modulates virulence associated Clp proteases that degrade misfolded proteins. Under stress conditions, CtsR repression is relieved and Clp proteases degrade damaged proteins, allowing the cell to tolerate stress. This ability is particularly important in escaping the phagolysosome and intracytoplasmic compartments (4, 30). Specifically, a Δ*ctsR* in EGD-e background was impaired in intracellular growth in P388D1 murine macrophage cell line as well as plaque formation, according to Chatterjee et al.,(4). Though CtsR was not detected as a

requirement for virulence-associated functions in our *in vitro* experiments, it is clearly important in other models. In light of this, it could be that our chosen cell culture experiments were limited in their detection of CtsR as an important regulator in virulence. It is also possible that CtsR may not be as important in *in vitro* cell culture models in the strain 10403S as it is in EGD-e because of heterogeneity of function across different strains. It may also be that our animal model better represented the true role of CtsR as compared to our tissue culture model. Interestingly, the *ctsR* gene itself has been shown to be moderately up regulated specifically in the vacuolar compartment of the cell (4). Furthermore, of the 42 genes repressed by CtsR (14), 15 are upregulated during infection in the mouse spleen (3). In line with this, a strain with inactive CtsR, whose repressor function is lost due to a single amino acid deletion, exhibits virulence attenuation in a mouse (16). It is possible that the *ctsR* mutant tested in the guinea pig could not escape vacuolar compartments to the cytoplasm to replicate and continue the intracellular life cycle in the animal model. Because of the attenuation of the Δ*ctsR* strain seen in our studies, these data point to the necessity of the negative regulator CtsR in establishing a full-scale persistent *L. monocytogenes* infection in a mammal.

CtsR is likely an important determinant in *L. monocytogenes* pathogenesis because it confers ability to adapt to stresses during transit through the intestinal tract (14), to overcome metabolic and nutritional limitations (14) and to withstand cytosolic and vacuolar stresses encountered in host cells (4, 30). While the other transcription factors did not play a demonstrable role in virulence and virulence-associated functions, it remains to be seen whether they play role indirect roles in virulence under other circumstances, such as infection of the brain or placenta. The observed phenotype may also be a result of overlapping regulation with other factors. The evolutionary necessity for a compensatory mechanism to respond and neutralize any

deleterious effects resulting from the loss of certain factors is a possible explanation for lack of phenotype. While some factors such as PrfA, σ^B, and CtsR have roles in pathogenesis, it is plausible that the effects of the remaining regulators may be masked by the complex circuitry of the transcriptional regulators as a whole. The ability of *L. monocytogenes* to utilize this sophisticated network of factors advantageously permits this bacterium to swiftly modulate cell responses under a variety of adverse circumstances allowing the organism to sense, respond and survive.

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CHAPTER 3

σ^B AND σ^L CONTRIBUTE TO L. MONOCYTOGENES 10403S RESPONSE TO THE ANTIMICROBIAL PEPTIDES SDPC AND NISIN[1]

ABSTRACT

The ability of the foodborne pathogen *Listeria monocytogenes* to survive antimicrobial treatments is a public health concern, therefore, this study was designed to investigate genetic mechanisms contributing to antimicrobial response in *L. monocytogenes*. In previous studies, the putative bacteriocin immunity gene *lmo2570* was predicted to be regulated by the stress responsive alternative sigma factor, σ^B. As the alternative sigma factor σ^L controls expression of genes important for resistance to some antimicrobial peptides, we hypothesized roles for *lmo2570*, σ^B, and σ^L in *L. monocytogenes* antimicrobial response. Results from phenotypic characterization of a *L. monocytogenes lmo2570* null mutant suggested that this gene does not contribute to resistance to nisin or to SdpC, an antimicrobial peptide produced by some strains of *Bacillus subtilis*. While *lmo2570* transcript levels were confirmed to be σ^B-dependent, they were σ^L-independent and were not affected by the presence of nisin under the conditions used in this study. In spot-on-lawn assays with the SdpC-producing *B. subtilis* EG351, the *L. monocytogenes* Δ*sigB*, Δ*sigL* and Δ*sigB*/Δ*sigL* strains all showed increased sensitivity to SdpC, indicating that both σ^B and σ^L regulate genes contributing to SdpC resistance. Nisin survival assays showed that σ^B and σ^L both affect *L. monocytogenes* sensitivity to nisin in broth survival assays, i.e., a *sigB* null mutant is more resistant than the parent strain to nisin, while a *sigB* null mutation in Δ*sigL* background leads to reduced nisin resistance. In summary, while the σ^B-

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dependent *lmo2570* does not contribute to resistance of *L. monocytogenes* to nisin or SdpC, both σ^B and σ^L contribute to the *L. monocytogenes* antimicrobial response.

INTRODUCTION

The Gram-positive, facultative intracellular foodborne pathogen *Listeria monocytogenes* is the causative agent of listeriosis, which has a human case-fatality rate > 20% in the U.S. (36). The vast majority of human listeriosis cases have been reported to occur via consumption of contaminated foods (36), therefore, development of more effective methods for controlling the presence of *L. monocytogenes* in foods is a desirable goal. To that end, various antimicrobial peptides have been investigated as a potential means for inhibiting growth of *L. monocytogenes* in foods (13, 39).

Bacteriocins are bacterially produced antimicrobial peptides that are generally most effective against other bacteria that are genetically similar and present in similar ecological niches. To enhance producer strain self-preservation, bacteriocin production is frequently coupled with production of cognate bacteriocin immunity proteins (13, 17, 45). For example, NisI, which provides immunity to nisin, is encoded downstream of the nisin biosynthesis genes in *Lactococcus lacti*s (19). Previous studies have reported that bacteriocin production can be influenced by bacterial environmental stress response pathways such as the RecA-dependent SOS response and the ppGpp-dependent stringent response (16). Bacteriocin production and immunity to antimicrobials are hypothesized to enhance the ability of producer bacteria to vie for limited nutrients in the presence of competitors (40).

Lactic acid bacteria (LAB) are recognized as producers of various bacteriocins (27, 30). LAB are commonly present in human foods, and therefore, the bacteriocins that they produce, such as pediocin PA-1/AcH, enterocins, and/or sakacins also may be present in foods (13). Currently, only nisin, a class I lantibiotic bacteriocin

produced by the lactic acid bacterium *Lactococcus lactis*, has Generally Recognized as Safe (GRAS) status for intentional application as an antimicrobial in the U.S. food industry (27). Nisin creates membrane-spanning pores in the bacterial cell wall, which enable dissipation of the cell's proton motive force (7, 8). Although, in general, nisin has been demonstrated as an effective antilisterial peptide (5), some strains of *L. monocytogenes* have developed resistance to both nisin and pediocin PA-1 (22-24). *L. monocytogenes* resistance to nisin is a concern to the segments of the food industry (e.g., dairy, poultry) that currently use this peptide to control pathogen growth (22). A better understanding of the molecular mechanisms contributing to antimicrobial resistance in foodborne pathogens could lead to development of improved food safety intervention strategies. One means to that end is to identify and examine putative bacteriocin immunity genes and their physiological roles in protecting the producer strain against either endogenously or exogenously produced antimicrobial peptides.

In a previous study, Kazmierczak et al. (28) identified *lmo2570* as a putative σ^B-dependent gene with 45% similarity to the *B. subtilis* bacteriocin immunity gene *sdpI* (*yvaZ*) (cmr.jcvi.org), which encodes SdpI. SdpI is a membrane protein conferring resistance to the endogenously-produced antimicrobial peptide SdpC (9, 18). Butcher and Helmann (2006) found that while SdpI has a predominant role in conferring resistance to SdpC, the *B. subtilis* regulon contolled by σ^W, an extracytoplasmic function sigma factor (ECF), provides secondary immunity to this antimicrobial peptide. Taken together, these data indicate the importance in antimicrobial resistance of both immunity genes and transcriptional level regulatory mechanisms as mediated by alternative sigma factors.

We hypothesized that σ^B and σ^L contribute to antimicrobial response in *L. monocytogenes*. σ^B has been shown to regulate response to antimicrobial peptides in other Gram-positive bacteria. To illustrate, the *B. subtilis* σ^B regulon is up-regulated

following treatment with either bacitracin or vancomycin (33). In a collection of teicoplanin-resistant *Staphylococcus aureus* mutants, the majority of the mutations responsible for antimicrobial resistance mapped to *rsbW*, which encodes the RsbW anti-sigma factor that sequesters σ^B to prevent it from interacting with RNA polymerase. The teicoplanin-resistant strains with mutations in *rsbW* showed increased σ^B activity relative to their parent strain (the MB33 *rsbU* mutant strain) or to other strains carrying the *rsbU* wild-type allele (6), providing evidence of a link between σ^B activity and antimicrobial resistance. σ^B also has been shown to contribute to bacterial stress response regulation in *Staphylococcus aureus* (10). *L. monocytogenes* alternative sigma factor σ^L regulates expression of genes that mediate sensitivity to antimicrobials such as the class IIa bacteriocin, mesentericin Y105 (42), hence σ^L also has been associated with antimicrobial response. Therefore, in the studies described below, phenotypic and genotypic assessments were used to determine the contributions of σ^B, σ^L and Lmo2570 to the *L. moonocytgenes* response to SdpC and nisin.

MATERIALS AND METHODS

Bacterial strains and growth conditions

L. monocytogenes parent strain 10403S (serotype 1/2a), and otherwise isogenic *sigB* and *sigL* single and double null mutants (Δ*sigB*; FSL A1-254, Δ*sigL*; FSL B2-124, Δ*sigB/*Δ*sigL*; FSL B2-127) were used in this study. *L. innocua* FSL C2-008 (47), *L. ivanovii* FSL C2-010, *L. welshimeri* FSL N1-064, and *L. seeligeri* FSL N1-067 (Table 3.1) were used to assess intragenus competition with the *L. monocytogenes* parent and mutant strains. To examine the susceptibility of the *L. monocytogenes* parent and mutant strains to a closely related bacterium that produces an antimicrobial peptide, we used strains of *B. subtilis* that produce SdpC, the bacteriocin whose

Table 3.1: Strains used in this study

Strain	Characteristics	Reference or source
L. monocytogenes 10403S	laboratory parent strain	Bishop and Hinrichs, 1987
L. monocytogenes FSL A1-254	10403S Δ*sigB*	Wiedmann et al., 1998
L. monocytogenes FSL P1-002	10403S Δ*lmo2570*	This study
L. monocytogenes FSL B2-124	10403S Δ*sigL*	Chaturongakul, unpublished
L. monocytogenes FSL B2-127	10403S Δ*sigB*/Δ*sigL*	Chaturongakul, unpublished
L. innocua FSL C2-008		Woodling and Moraru, 2005
L. ivanovii FSL C2-010		Wiedmann, unpublished[a]
L. welshimerii FSL N1-064		Fish processing plant environment
L. seeligeri FSL N1-067		Fish processing plant environment
B. subtilis PY79	prototroph, parent strain	Youngman et al., 1984
B. subtilis EG351	PY79 $_{Pspac}$-hy-*sdpABC*	Butcher and Helmann, 2006

[a]Isolate kindly provided (as USDA 2717) by I. Wesley, USDA-ARS

cognate immunity gene is predicted by sequence similarity to be homologous to *L. monocytogenes lmo2570*. These strains included *B. subtilis* prototroph (PY49) (48) and its mutant EG351 (PY79 P_{spac}-hy-*sdpABC*) (gift of Dr. J. Helmann, Dept of Microbiology, Cornell University), which expresses SdpC under control of an inducible promoter.

L. monocytogenes strains were grown in brain heart infusion broth (BHI; Difco, Sparks, MD) at 37ºC with shaking (250 rpm) overnight (16-18 h), then were subcultured (1:100) and grown as described below for each experiment. *B. subtilis* strains were grown in Luria-Bertani (LB) broth as described for *L. monocytogenes*, unless otherwise stated. The Δ*sigB*/Δ*sigL* strain grew more slowly than the 10403S, Δ*sigB*; and Δ*sigL* strains, requiring an additional incubation time of ~30 minutes to reach the same OD_{600}.

Mutant construction

An in-frame 543 base pair deletion within *lmo2570* was created in *L. monocytogenes* 10403S using splicing-by-overlap extension (SOE) PCR and allelic exchange mutagenesis (25). Primers used were 5'-GGA AGC TTT AAG GCA CTG TGA GCC TGG-3' (*lmo2570* SOEA), 5'-TCA TAC TAG GAA ATA TAC CAA C-3'(*lmo2570* SOEB), 5'-GTT GGT ATA TTT CCT AGT ATG ATT ATT GTT GTT G-3'(*lmo2570* SOEC), 5'-GGG GTA CCT CAG GTT CAC TGG CAG CTA G-3' (*lmo2570* SOED). Primers were synthesized by IDT Technologies (Coralville, IA). Allelic exchange mutagenesis was confirmed through PCR and subsequent DNA sequencing, the latter of which was performed by the Cornell BioResource Center (Ithaca, NY). The Δ*lmo2570* mutation did not affect growth rate of the mutant strain relative to the 10403S parent strain when both were grown in BHI at 37ºC with shaking at 250 rpm (data not shown).

Spot-on-lawn Assays

Spot-on-lawn assays were performed in triplicate as described by (9). Briefly, to create lawns, 100µl of a given strain, (i.e., 10403S, Δ*lmo2570*, Δ*sigB*, Δ*sigL*, or Δ*sigB*/Δ*sigL*) that had been grown to an optical density of OD_{600}= 0.4 was inoculated into 2 ml of 0.7% LB soft agar that had been tempered at 50ºC. To induce P_{spac}-regulated transcription of *sdpABC* when EG351 was used as the spotting strain, 1mM isopropyl-β-D-thiogalactopyranoside (IPTG) also was added to the tempered agar that had been inoculated with bacteria. Each mixture was then poured into one well in an 8-well rectangular multidish (26mm x 33mm; Nunc, Rochester, NY). The plates were then dried in a laminar hood for 30 minutes. Subsequently, 4µl of the strain being assessed for bacteriocin production (e.g., 10403S, PY79 or EG351), which had been grown to an OD_{600}= 0.6, was spotted on the agar in the middle of each well. The plates were covered with lids and incubated in a moist container at 37ºC for 22-24 hours. In addition to the 10403S, PY79 or EG351 test strains, isolates representing 5 *Listeria* species also were used as spotting strains to determine if the lawn strains would demonstrate sensitivity to bacteria representing different species within the same genus (Table 3.1). Sensitivity of lawns to potential bacteriocin producer strains was assessed by measuring the zone of inhibition (zoi) around the growth of the spotted strain. Radii of zoi were determined by measuring the diameters of both the spotted colony and the surrounding zoi in pixels (px). The diameter of the spotted colony was then subtracted from the diameter of the zoi and the resulting product was divided by 2 (to yield a radius). Measurements were performed using Adobe® Photoshop® CS (Adobe Systems Incorporated, Mountain View, CA.).

Radii of zoi produced on the various lawn strains were initially compared to zoi produced on the 10403S reference lawn using one-way ANOVA with Dunnett's t-test, using SAS® 9.0 (SAS Institute, Inc., Cary, NC). To determine if there were

statistically significant interaction effects between the *sigB* and the *sigL* deletions, a two-way ANOVA (with Tukey's adjustment for multiple comparisons) was performed. In this model, the dependent variable was zoi radius, the independent variables included sigB + sigL + sigB*sigL + replicate. The factors "sigB" and "sigL" in the model indicate the presence or absence of that gene in the strains tested. An adjusted $p<0.05$ was considered significant in this and all other statistical analyses.

Nisin MIC determination

The objective of this experiment was to determine a minimal inhibitory concentration of nisin for log phase *L. monocytogenes* to enable selection of an appropriate sub-lethal concentration for subsequent qRT-PCR experiments. Nisin's solubility and activity are optimal at pH 3 and 3.5, respectively (1), therefore, nisin is typically dissolved in an acidified solution prior to use (26, 31). As σ^B expression and activity are induced at low pH (3, 44), we predicted that addition of nisin in an acidified solution to the various cultures would up-regulate expression of the σ^B regulon, thus conferring a survival advantage to the wildtype over the Δ*sigB* strain (20, 21, 46) and, hence, confounding interpretation of our experimental results. Therefore, to avoid induction of σ^B activity, nisin was dissolved in sterile distilled water (1000AU/ml) and the pH of the final solution was adjusted to 7.0 using 0.01N sodium hydroxide. Nisin solutions at pH 7.0 were used throughout these experiments. The nisin solutions were filter sterilized with a 0.2µm, 25mm syringe filter (NALGENE®, Thermo Fisher Scientific, Waltham, MA) and diluted to the test concentrations. The minimum inhibitory concentration (MIC) of nisin (Sigma; St. Louis, MO) was determined for all strains of *L. monocytogenes* by measuring absorbance at OD_{600} using a Fusion™ Universal Microplate Analyzer (PerkinElmer; Shelton, CT). Strains were grown overnight, subcultured 1:100, and grown to $OD_{600}=0.4$. Strains were inoculated to a final concentration of $1x10^4$ CFU/well into 96-well round bottom

microplates (Costar, Corning, NY) and the edges were sealed with Parafilm® (Alcan Packaging; Neenah, WI) to prevent evaporation. OD_{600} measurements were taken following 24 h incubation at 37ºC with shaking. The lowest concentration that inhibited growth for all strains after a 24 hr incubation in a 96 well plate format was 100AU nisin/ml, as determined in three replicate trials. Therefore, a sub-lethal concentration of 75AU nisin/ml was used for TaqMan qRT-PCR assays.

Total RNA isolation

L. monocytogenes 10403S and Δ*sigB* were grown to logarithmic phase (OD_{600}=0.4) and collected after (i) incubation for 10 min following addition of nisin in sterile distilled water to yield a final concentration of 75AU/ml nisin; (ii) incubation for 10 min following addition of an equivalent volume of sterile distilled water without nisin; (iii) incubation for 10 min without any addition. RNA isolation and purification was performed as previously described (41, 44), except that DNase treatments were performed using TURBO™ DNase (Ambion, Austin, TX) following the manufacturer's instructions. Total nucleic acid concentrations and purity were estimated using absorbance readings (260 nm/280 nm) on a NanoDrop™ ND-1000 spectrophotometer (NanoDrop Technologies, Rockland, DE).

Quantitative Reverse-Transcriptase PCR (qRT-PCR)

Transcript levels of *lmo2570*, as well as of two housekeeping genes, *rpoB* and *gap*, were quantified using TaqMan primers and probes and the ABI Prism® 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) as previously described (29). Data were analyzed using the ABI Prism® 7000 Sequence Detection System (SDS) software (Applied Biosystems) as previously described by Sue et al. (44). Primer Express® 1.0 software (Applied Biosystems) was used to design oligonucleotide primers and TaqMan probes for *lmo2570*: forward primer (5'- AAG TGG CGG TGC ATT TCG-3'), reverse primer (5'-TAA GCC AAG CCA CTT TTG

CAT-3'), probe (6FAM 5'-ACG GAC TTC TCC CCA GAT-3' MGB-NFQ). Primers and probes for *gap* and *rpoB* were previously described (43, 44), respectively.

Transcript levels of *lmo2570,* as determined by qRT-PCR, were $\log_{10}$ transformed and then normalized to the geometric mean of transcript levels from the housekeeping genes *rpoB* and *gap* as previously described (29). Statistical analyses of normalized *lmo2570* transcript levels were performed using one-way ANOVA and Tukey's studentized range (HSD) test, performed in SAS® 9.0 (SAS Institute).

Nisin survival assay

The objective of this assay was to measure relative survival characteristics of stationary phase *L. monocytogenes* 10403S, Δ*sigB,* Δ*sigL,* Δ*sigB/*Δ*sigL,*and Δ*lmo2570* strains in the presence of an initially lethal concentration of nisin (150AU/ml nisin). Strains were grown in BHI at 37°C with shaking overnight, followed by a 1% subculture and growth to logarithmic phase (OD_{600}=0.4), followed by a second subculture and growth to stationary phase (OD_{600}=1.0 +3 hours), followed by a third 1% subculture (0.5ml into 50ml, final concentration of ~$2x10^7$ CFU/ml) in a 300 ml flask (Bellco, Vineland, NJ). Nisin (150 AU/ml, pH 7.0) was added to the BHI and cultures were incubated at 37°C with shaking for an additional 9 h. Bacterial numbers were determined prior to and after the addition of nisin. Specifically, samples were taken at 0, 0.5, 1, 2, 3, 4, 5, 6, and 9 h post-addition and spiral plated on brain heart infusion (BHI) agar using a Spiral Biotech Autoplate® 4000 (Spiral Biotech; Norwood, MA). Colonies were enumerated with a QCount™ (Spiral Biotech) after 24 h incubation at 37°C. Colony counts were transformed to $\log_{10}$ CFU/ml.

Data from the nisin survival assay were used to calculate two parameters: (i) bacterial reduction after 0.5 h of nisin exposure; and (ii) growth rate during recovery and re-growth (between 1 and 9 h after nisin exposure). Linear regression was used to determine the slope representing the change in bacterial numbers for each strain from

1 h to 9 h (i.e., the period when viable cell numbers were increasing [re-growth]); this value represents the bacterial growth rate in $\log_{10}$ growth/h. Statistical analyses were then performed on both parameters. First, a one way ANOVA (with Dunett's t-test or Tukey's studentized range [HSD] test) was performed to determine if (i) bacterial reduction or (ii) growth rate differed between the mutant strains and the parent strain. To determine if there were statistically significant interaction effects between the *sigB* and the *sigL* deletions, a two-way ANOVA (with Tukey's adjustment for multiple comparisons) was performed. In this model, the dependent variable was either (i) bacterial reduction after nisin exposure or (ii) growth rate during re-growth; the independent variables included sigB + sigL + sigB*sigL + replicate. The factors "sigB" and "sigL" in the model indicate the presence or absence of that gene in the strains tested.

RESULTS

No intragenus competition was evident between *L. monocytogenes* and the other *Listeria* strains tested (Table 3.1). Specifically, no zones of inhibition occurred between any of the listerial species that were used as spotting strains (*L. innocua*, *L. ivanovii*, *L. welshimeri*, or *L. seeligeri*) and any of the *L. monocytogenes* lawn strains (10403S, Δ*sigB*, Δ*sigL* or Δ*sigB*/Δ*sigL*; data not shown).

***lmo2570* is σ^B, but not σ^L dependent and does not contribute to resistance to nisin or SdpC**

qRT-PCR was initially used to determine whether either σ^B or σ^L contributes to transcription of *lmo2570*, a putative bacteriocin immunity gene (Figure 3.1). *lmo2570* transcript levels were consistently and significantly lower in the Δ*sigB* strain as compared to the 10403S parent strain ($p<0.05$; Figure 3.1), indicating σ^B-dependent

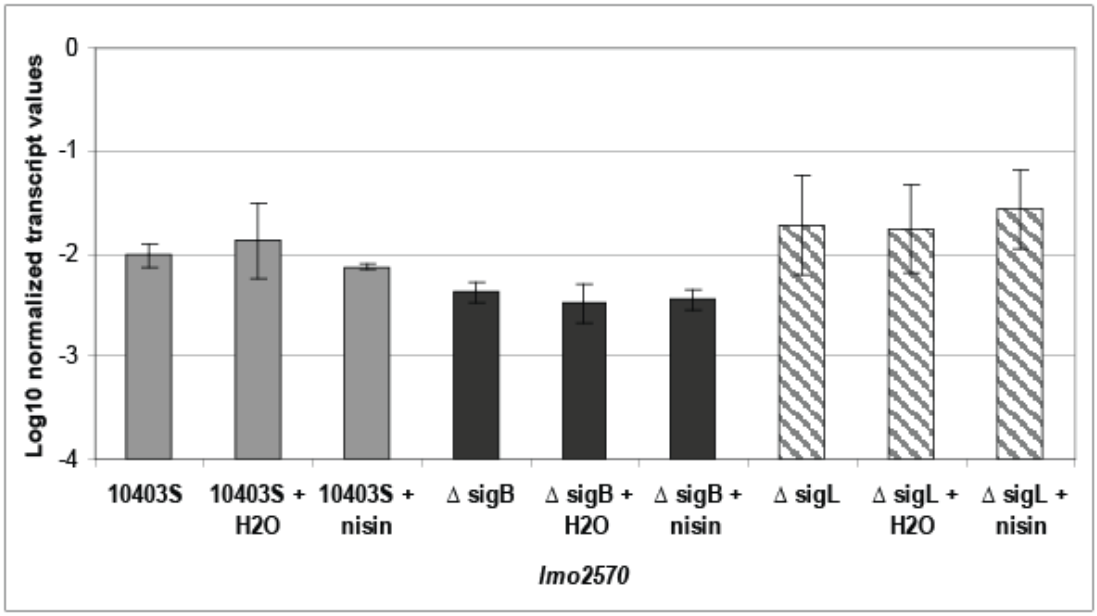

Figure 3.1: Normalized log transformed *lmo2570* transcript levels for *L. monocytogenes* 10403S (grey bars), Δ*sigB* (black bars) and Δ*sigL* (hatched bars). Transcript levels were determined by qRT-PCR using RNA isolated from logarithmic phase (OD_{600}= 0.4) *L. monocytogenes* that had been: (i) incubated for 10 min following addition of nisin in sterile distilled water to yield a final concentration of 75AU/ml nisin; (ii) incubated for 10 min following addition of an equivalent volume of sterile distilled water without nisin; or (iii) incubated for 10 min without any addition. Transcript levels were log transformed and normalized to the geometric mean of the transcript levels for the housekeeping genes *rpoB* and *gap*. Values represent mean transcript levels from three independent RNA collections; error bars indicate one standard deviation from each mean. Overall ANOVA (GLM) showed a significant effect of the factor "strain", but no effect of the factor condition (i.e., "no addition", "addition of water", or "addition of nisin"). Tukey's test showed significantly lower transcript levels for *lmo2570* in the □*sigB* strain as compared to the parent strain; transcript levels did not differ significantly between the □*sigL* strain and the parent strain.

transcription of *lmo2570*. While *lmo2570* transcript levels were consistently higher in the Δ*sigL* strain as compared to the parent strain, this difference was not significant ($p>0.05$; Figure 3.1). The presence of nisin at a sub-lethal concentration (75 AU/ml) did not affect *lmo2570* transcript levels (Figure 3.1). Normalized *lmo2570* transcript levels in 10403S were low, ranging from 0.007 to 0.034. To put these low *lmo2570* transcript levels into biological context, in 10403S, ~0.02 transcripts of *lmo2570* were present relative to the mean transcript levels of the highly expressed housekeeping genes, *rpoB* and *gap*, as indicated in Figure 3.1. The *lmo2570* transcript levels observed in the present study are on the same order of magnitude as those reported previously for other σ^B-dependent genes (e.g., *opuCA* and *bsh* (11)).

Spot-on-lawn assays were used to compare the sensitivities of *L. monocytogenes* 10403S and Δ*lmo2570* to SdpC, the antimicrobial peptide whose cognate immunity gene shares amino acid similarity with Lmo2570. Specifically, *B. subtilis* PY79 (which naturally produces SdpC) and *B. subtilis* EG351 (which overexpresses SdpC in the presence of IPTG) were spotted on lawns of either 10403S or Δ*lmo2570*. Zones of inhibition for 10403S and Δ*lmo2570* (Table 3.2) did not differ significantly ($p>0.05$), indicating that *lmo2570* does not contribute to SdpC resistance. Neither 10403S nor Δ*lmo2570* showed inhibition by 10403S (Table 3.2) or by any other *Listeria* species (data not shown), indicating absence of intragenus inhibition, at least among the strains tested.

To characterize the responses of stationary phase *L. monocytogenes* 10403S and Δ*lmo2570* to nisin, we evaluated survival of ~ 2 X 10^7 CFU/mL 10403S or Δ*lmo2570* in BHI in the presence of 150 AU nisin /ml (Figure 3.2). Exposure to nisin for 30 min led to 4.0 and 3.9 log reductions in bacterial numbers for 10403S and Δ*lmo2570*, respectively (Figure 3.2), indicating no difference in nisin susceptibility between these strains. After the initial killing by nisin, bacterial numbers increased

Table 3.2: Spot-on-lawn assay results

Strain spotted[a]	Mean zone of inhibition radii (SD) for *L. monocytogenes* strains[b]				
	10403S	Δ*lmo2570*	Δ*sigB*	Δ*sigL*	Δ*sigB*/Δ*sigL*
L. monocytogenes 10403S	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
B. subtilis PY79	11.8 (1.6)	10.0 (2.2)	11.5 (4.9)	16.7 (0.6)	20.5* (2.3)
B. subtilis EG351 (IPTG)	13.5 (3.9)	8.3 (2.9)	28.2* (1.6)	20.7* (5.3)	32.0* (2.6)

[a] Strains spotted on the lawns are listed in left column; the average zoi radius around each spot is shown for each lawn, with standard deviations (SD) in parentheses.

[b] Radii were determined from three independent experiments by measuring diameters of the zones of inhibition (zoi) in pixels using Adobe® Photoshop® CS; "*" indicates values that are significantly different ($P<0.05$; one way ANOVA with Dunnett's t-test) from the zoi produced on the *L. monocytogenes* 10403S lawn

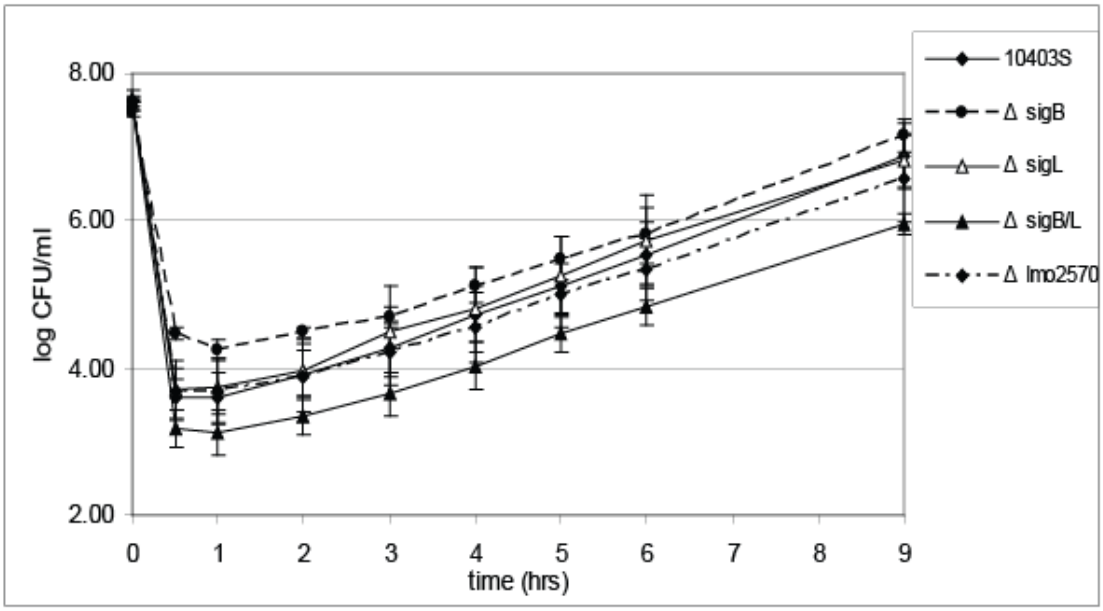

Figure 3.2: Viable numbers of stationary phase *L. monocytogenes* 10403S, Δ*lmo2570*, Δ*sigB*, Δ*sigL* and Δ*sigB*/Δ*sigL* at various time points following exposure to 150 AU/ml nisin. Values are reported as log CFU/ml. Data shown represent the average of four independent experiments; error bars represent one standard deviation from each mean.

between 1 and 9 h post-nisin exposure, reflecting growth of cells that had survived nisin exposure. Specifically, after 9 h, bacterial numbers were 6.9 and 6.6 log for the parent strain and Δ*lmo2570*, respectively, further supporting that Δ*lmo2570* and 10403S susceptibilities to nisin do not differ.

σ^B and σ^L both contribute to resistance to the SdpC antimicrobial peptide produced by *B. subtilis*

Spot-on-lawn assays were used to compare the sensitivities of *L. monocytogenes* 10403S, Δ*sigB*, Δ*sigL*, and Δ*sigB*/Δ*sigL* to the antimicrobial peptide SdpC. The zones of inhibition (zoi) produced by *B. subtilis* PY79 did not differ between *L. monocytogenes* 10403S and Δ*sigB* or Δ*sigL*; the zoi produced by PY79 on the Δ*sigB*/Δ*sigL* lawn was significantly larger than the zoi produced on 10403S ($p<0.05$; Table 3.2), suggesting the possibility that σ^B and σ^L contribute to SdpC resistance in an additive fashion. *B. subtilis* EG351 produced significantly larger zoi on Δ*sigB* (28.2 ± 1.6 px), Δ*sigL* (20.7 ± 5.3 px) and Δ*sigB*/Δ*sigL* lawns (avg 32.0 ± 2.6 px), as compared to the zoi produced on 10403S (13.5 ± 3.9; Table 3.2). Two-way ANOVA analyses of zoi data showed no significant "sigB*sigL" interaction effect on SdpC sensitivity ($p>0.05$), further supporting the notion of additive (as compared to multiplicative) contributions of σ^B and σ^L to SdpC resistance. Overall, results from this assay indicate that alternative sigma factors σ^B and σ^L both contribute to resistance to SdpC.

σ^B and σ^L both contribute to response to the bacteriocin nisin

A 30 min exposure to nisin (150AU/ml) resulted in a 4.0 log reduction in bacterial numbers for stationary phase 10403S. By comparison, reduction of Δ*sigB* bacterial numbers was significantly less (3.0 log reduction; $p<0.05$; Figure 3.2),

indicating increased nisin resistance of this strain relative to that of 10403S. Bacterial numbers for the Δ*sigL* and Δ*sigB*/Δ*sigL* strains were reduced by 3.9 and 4.5 log; these reductions were not significantly different from that of 10403S ($p>0.05$; Dunnett's t-test). Interestingly, log reduction for the Δ*sigB*/Δ*sigL* strain was significantly ($p>0.05$; Tukey's HSD) greater (4.5 log) as compared to the Δ*sigB* strain (3.0 log reduction), indicating a significant effect of the *sigL* deletion on nisin killing in a Δ*sigB* background. Based on the reductions in bacterial numbers between 0 and 0.5 h, two-way ANOVA analyses found a significant "sigL*sigB" interaction effect on survival following nisin exposure, indicating that the effect of one sigma factor on survival differs depending on the presence or absence of the other sigma factor.

After initial killing by nisin, all strains showed re-growth between 1 and 9 h post nisin exposure. Rates of re-growth, as represented by the slopes of the graphs between 1 and 9 h post-exposure, were compared among the strains. While the growth rate for the Δ*sigL* strain (0.39 log CFU/h) was not significantly different from that of the parent strain (0.41 log CFU/h), both the Δ*sigB* and the Δ*sigB*/Δ*sigL* strains showed significantly slower growth rates (0.36 log CFU/h for both; $p<0.05$, Dunett's t-test) as compared to the parent strain. Although no significant "sigB*sigL" interaction effect on nisin survival was identified by two-way ANOVA analysis, the *sigB* deletion had a significant effect on growth rate, with s*igB* deletion strains (i.e., Δ*sigB*, Δ*sigB*Δ*sigL*) showing slower growth rates as compared to the corresponding strains with intact *sigB* genes.

DISCUSSION

We hypothesized that alternative sigma factors σ^B and σ^L and the hypothetical bacteriocin immunity gene, *lmo2570*, contribute to *L. monocytogenes* antimicrobial response. This hypothesis was based on previous observations including: (i) σ^B and its

homolog σ^F contribute to antimicrobial response in other Gram-positive organisms (6, 33, 37), (ii) *L. monocytogenes* σ^L controls sensitivity to class IIa bacteriocins, mesentericin Y105, pediocin PA-1, and enterocin A, and (iii) the putative σ^B-dependent *lmo2570* has sequence homology to the *B. subtilis* bacteriocin immunity gene, *sdpI*. To test our hypothesis, we assessed the sensitivities of strains with null mutations in *sigB*, *sigL*, *sigB/sigL*, or *lmo2570* to the antimicrobial peptides SdpC and nisin. We also characterized transcription of *lmo2570* in 10403S, Δ*sigB*, and Δ*sigL* strains exposed to a sub-minimal inhibitory concentration of nisin. Our results show that (i) while *lmo2570* is σ^B-dependent, it does not contribute to resistance to SdpC or nisin; and (ii) both σ^B and σ^L contribute to resistance to the antimicrobial peptide SdpC, as shown by results from spot-on-lawn assays. In addition, both σ^B and σ^L affect *L. monocytogenes* sensitivity to nisin in broth survival assays. Specifically, while loss of only *sigB* renders the resulting strain more resistant to nisin than the parent strain, loss of *sigB* in a Δ*sigL* background leads to reduced nisin resistance relative to the original parent strain.

The effects of antimicrobial peptides on *L. monocytogenes* appear to differ depending on the class of peptide, the strain, initial number of bacteria, growth phase, and the assay used for evaluation. To illustrate, σ^B was reported previously to contribute to *L. monocytogenes* tolerance to nisin or lacticin 3147 in broth assays (4), but not to nisin, lacticin 3147, or sakacin A resistance in agar overlay assays (4, 38). Moorhead and Dykes (2003) showed that a *L. monocytogenes* serotype 1/2a wildtype strain was less resistant to nisin than a serotype 4c wildtype strain, suggesting differences in antimicrobial sensitivities among strains. It is also likely that other environmental stresses (in addition to the presence of the antimicrobial peptide) imposed upon the cells also evoke differential phenotypic responses from the cells

(e.g., exposure to low pH induces σ^B activity in *L. monocytogenes*), which may provide cross-resistance to multiple stresses (21).

lmo2570 is σ^B-dependent, but does not contribute to antimicrobial resistance

L. monocytogenes Lmo2570 is 45% similar at the amino acid level to the *B. subtilis* immunity protein SdpI, which confers immunity against SdpC (9, 18), therefore, we hypothesized that *lmo2570* may play a role in antimicrobial immunity in *L. monocytogenes*. *lmo2570* was predicted as σ^B-dependent in previous microarray experiments (28). As previous reports have shown bacteriocin immunity genes can contribute to resistance to multiple antimicrobials (35), in addition to examining its role in SdpC resistance, we also tested the contributions of *lmo2570* to resistance to the commercially available bacteriocin, nisin. The Δ*lmo2570* strain did not show reduced sensitivity to either SdpC or nisin. Exposure to nisin did not induce transcription of *lmo2570* in either the wildtype or any of the mutant strains. *lmo2570* thus does not appear to be important for SdpC or nisin resistance in *L. monocytogenes*. A role for this gene in resistance to other bacteriocins or in contributing to nisin and SdpC resistance under environmental conditions not tested here cannot be excluded by our data, however. Our confirmation of *lmo2570* as σ^B-dependent suggests a role for *lmo2570* in *L. monocytogenes* survival or growth under conditions that remain to be defined.

σ^B and σ^L both contribute to *L. monocytogenes* response to SdpC and nisin

We found clear evidence that alternative sigma factors σ^L and σ^B both contribute to SdpC resistance. Specifically, as determined in a spot-on-lawn assay, we showed that both the Δ*sigB* and the Δ*sigL* strains were significantly more susceptible to the bactericidal effect of the antimicrobial peptide SdpC produced by *B. subtilis*

EG351 than the otherwise isogenic 10403S parent strain. Characterization of a Δ*sigB*/Δ*sigL* double mutant strain suggested that deletion of both genes had an additive, but not an interactive, effect on SdpC resistance. However, deletions of both Δ*sigB* and Δ*sigL* had an interactive effect on *L. monocytogenes* resistance to nisin. Specifically, while the Δ*sigB*/Δ*sigL* strain showed decreased resistance to nisin as compared to the Δ*sigB* strain, the Δ*sigB* strain showed increased resistance to nisin as compared to the parent strain, which has both *sigB* and *sigL* intact. The interactive effect observed following the loss of both *sigB* and *sigL* may indicate that at least some genes important for recovery and re-growth following nisin exposure are co-regulated, either directly or indirectly, by these alternative sigma factors. We also found that re-growth of both the Δ*sigB* and the Δ*sigB*/Δ*sigL* strains following nisin exposure was slower than that of the parent strain, consistent with previous observation that σ^B is important for *B. subtilis* recovery following rifampin treatment (2). The overall observation that a deletion of the gene encoding σ^L (i.e., a single deletion) does not affect *L. monocytogenes* resistance to nisin is consistent with observations by (15), who reported that σ^L (which has also been designated as RpoN) is not involved in *L. monocytogenes* nisin resistance. Relative to its otherwise isogenic parent, a Δ*rpoN L. monocytogenes* strain (i.e., a strain lacking σ^L) has previously shown increased resistance to the class IIa nonlantibiotic bacteriocins mesentericin Y105, pediocin PA-1, and enterocin A (15, 42), consistent with our findings that σ^L contributes to resistance to some bacteriocins (i.e., SdpC). Our findings, as well as previous findings by others (15), thus support that different regulatory elements are critical for the ability of *L. monocytogenes*, and other bacteria, to respond to different bacteriocins, a notion consistent with the diverse nature of this group of antibacterial compounds.

Overall, our data indicate that σ^B and σ^L both contribute to the ability of *L. monocytogenes* to respond to antimicrobials. Regulatory interactions among multiple alternative sigma factors also have been shown to contribute to antibiotic resistance in *B. subtilis*. Specifically, three (σ^M, σ^W, and σ^X) of the seven *B. subtilis* ECF alternative sigma factors have overlapping regulons that contribute to antibiotic resistance, as demonstrated by the greatly enhanced sensitivity of a triple MWX mutant to various antimicrobials, including nisin (32). Strains bearing single or double mutations in the genes encoding these alternative sigma factors displayed considerably less antimicrobial sensitivity than the strain with the triple mutation (32). Thus, in combination with previous studies, our data support a model in which multiple alternative sigma factors contribute to regulatory networks important for fine-tuning transcriptional regulation of gene expression to help optimize bacterial cell resistance to antimicrobial peptides.

CONCLUSIONS

Alternative sigma factors have been shown to regulate genes and operons critical for resistance to antimicrobials in various bacteria, including *B. subtilis*, *L. monocytogenes*, *Salmonella enterica* serovar Typhimurium, *S. aureus*, and *Vibrio cholerae* (9, 14, 34, 42, 49). Our data indicate that σ^B and σ^L, as well as the simultaneous presence of both σ^B and σ^L, contribute to antimicrobial response in *L. monocytogenes* in a manner that is dependent on the antimicrobial that is present. The results reported in this study provide further evidence of the importance of regulatory networks for fine-tuning *L. monocytogenes* responses to changing environmental conditions (12).

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CHAPTER 4

IDENTIFICATION OF A SMALL MOLECULE THAT INHIBITS THE *L. MONOCYTOGENES* σ^B REGULON AND ITS VIRULENCE ASSOCIATED FUNCTIONS

ABSTRACT

For some bacteria, current treatment options are minimally effective. In fact, for *Listeria monocytogenes*, which causes the rare but potentially deadly disease listeriosis, the mortality rate remains at 20-30% despite the use of antibiotics. This indicates the need for the identification of new drug targets for increasing efficacious treatment alternatives. The *L. monocytogenes* alternative stress response sigma factor σ^B represents a conserved biological target, highly relevant to several important Gram-positive human pathogens. In these pathogens, σ^B regulates virulence genes and contributes to survival under host-associated stress conditions, such as those encountered in the gastrointestinal lumen. Using a high-throughput cell-based format to identify inhibitors of *L. monocytogenes* σ^B, approximately 57,000 compounds were screened from a compilation of natural and synthesized small molecules. Subsequent screening identified a compound on which transcriptional and phenotypic profiling were performed. The compound, sigmastatin (IC_{50}=3.5μM), showed targeted down regulation of the majority of the σ^B regulon yielding a transcriptional profile similar to a genetic knockout of *sigB*. From the genes downregulated by sigmastatin, 75% were σ^B dependent. Specifically, according to microarray analysis, of the 208 genes that were downregulated as a result of treatment with this compound, 156 were positively regulated by σ^B, including key virulence and stress response genes such as *inlA, inlB, bsh, hfq, opuC, bilE*. This compound also hinders *L. monocytogenes* invasion in human intestinal epithelial cells. Interestingly, this small molecule was also capable of

inhibiting σ^B activity in *Bacillus subtilis*. The ability of sigmastatin to produce a chemical knockout phenotype comparable to a genetic knockout supports its usefulness as a biological probe. Not only does this allow us to explore the molecular underpinnings of *L. monocytogenes* that drive virulence and stress response, but it also helps to elucidate complex regulatory networks in order to develop better methods to control this pathogen.

INTRODUCTION

Listeria monocytogenes is the causative agent of a rare, but potentially fatal, foodborne disease called listeriosis. Listeriosis has a high case fatality rate, accounting for ~10% of all deaths from foodborne diseases in the US (61). According to the Centers for Disease Control, in 2008 the incidence of listeriosis infections had not declined in the US in the preceding three years (1). Furthermore, there has been an increasing incidence of listeriosis in Europe since 2004 (33). These data suggest the need for development of more effective preventive strategies and interventions.

L. monocytogenes can transition from a saprotrophic existence under a wide range of environmental conditions (70) to intracellular infection in a diverse array of hosts (101). The ability of *L. monocytogenes* to transform from saprotroph to intracellular pathogen is influenced by regulatory networks that control virulence factor expression in response to environmental signals (18). σ^B is one important component of a network that links environmental stress survival and virulence in *L. monocytogenes* (72, 98). σ^B networks contribute to transmission of *L. monocytogenes*, including during the gastrointestinal and systemic stages of infection (14, 30).

Sigma (σ) factors are dissociable subunits of prokaryotic RNA polymerase. The association of a specific alternative sigma factor (such as σ^B) with core RNA polymerase under appropriate environmental conditions reflects a transcriptional

regulatory mechanism that can rapidly reprogram global gene expression patterns in response to environmental signals. Through microarray analyses, the σ^B regulon in *L. monocytogenes* has been reported to include >150 genes (45, 77). Because of its key role in *L. monocytogenes* stress resistance and virulence, and hence, in transmission of this pathogen, σ^B is a promising target for investigation and development of novel therapeutic intervention strategies.

Identification of novel anti-infective agents by screening small-molecule libraries for inhibitors or perturbational agents of specific targets is one promising approach for development of new therapeutics. Such strategies have been used to identify inhibitors of virulence-related two-component regulators and quorum sensing in *Pseudomonas aeruginosa* (36, 37, 83), inhibitors of the Type 3 Secretion System in multiple Gram-negative bacteria (3, 44, 69), inhibitors of anthrax lethal factor in *Bacillus anthracis* (41, 75, 84), and inhibitors of the virulence regulator ToxT in *Vibrio cholerae* (40), among others. To test the hypothesis that a small molecule can inhibit σ^B activity, a high-throughput assay was used to screen multiple small-molecule libraries. The ability of the most promising compound to inhibit σ^B activity was further assessed by small molecule binding microarray analysis, whole genome microarray, qRT-PCR, and phenotypic profiling, including bile salt hydrolase activity and Caco-2 cell invasion assays. Further, the compound was assessed for its ability to inhibit σ^B activity across genera.

MATERIALS AND METHODS

Strain and media selection

As *L. monocytogenes opuCA* transcription has been clearly established as σ^B-dependent (45, 97), an *opuCA-gus* reporter fusion was selected for monitoring σ^B activity. The strains used in this study included the *L. monocytogenes* parent strain

10403S (serotype 1/2a)(7), its otherwise isogenic *sigB* mutant derivative (Δ*sigB*; FSL A1-254 (102)), a reporter strain for σ^B activity (10403S *opuCA-gus*; FSL S1-063) and a negative control reporter strain for σ^B activity (Δ*sigB opuCA-gus*; FSL S1-059) (Table 4.1). To achieve low background fluorescence, a chemically defined minimal medium (76) with 25mM glucose (DMG) (26) was used for the high-throughput screen. Cells were grown in brain heart infusion broth (BHI; Difco, Sparks, MD) for phenotypic and transcriptional profiling assays.

High-throughput Cell-Based Small Molecule Screen

Primary Cell Based Screen

The *L. monocytogenes opuCA-gus* fusion strain FSL S1-063 was used in a cell-based high-throughput screen (HTS) against ~ 57,000 compounds. As reported at *ChemBank.Broad.Harvard.edu*, compounds came from a multitude of libraries including libraries of (i) known bioactive compounds (i.e., SPBio, SMP libraries); (ii) synthetic compounds from diversity oriented synthesis (i.e., CMLD, ICCB, PK04, Ald1.1-H, Sulf1.1-A libraries); (iii) natural products (i.e., PhilEx, ICBGEx libraries), and (iv) pharmaceuticals.

Multidrop liquid handling robots (Matrix, ThermoFisher) were used to dispense 27μl of DMG into black walled clear bottom 384-well plates (Nunc; Rochester, NY), then 100nl of ~10mM stock of each small molecule was transferred from library stock or source plate to assay plates in a total volume of 30μl (e.g., 8009-2163 had a stock concentration of 19.3mM, producing a 64.3μM final concentration in the well) by the CyBi®-Well Vario pin transfer robot (CyBio AG; Jena, Germany). Each source plate contained ~15 dimethyl sulfoxide (DMSO)-only wells as negative internal control wells. All source plates were pinned in duplicate to provide experimental replicates (i.e., plates A and B). Two DMSO base plates were also included as external plate controls. A custom assay plate containing 192 wells of

Table 4.1: Strains used in this study

Strain	Characteristics	Reference
10403S	Laboratory Parent Strain	Bishop and Hinrichs, 1987
FSL A1-254 (LM Δ*sigB*)	Control strain, complete inhibition of σ^B activity	Wiedmann et al., 1998
FSL S1-063 (LM *opuCA-gus*)	Reporter strain for σ^B activity in *L. monocytogenes*	Ferreira et al., 2003
FSL S1-059 (LM Δ*sigB opuCA-gus*)	Negative control reporter strain for σ^B activity in *L. monocytogenes*	Ferreira et al., 2003
FSL P1-015 (BS PB198 *amyE::pDH32-ctc trpC2*)	Reporter strain for σ^B activity in *B. subtilis*	Boylan et al., 1992
FSL P1-017 (BS PB345 *amyE::pDH32-ctc sigBΔ3::spc trpC2*)	Negative control reporter strain for σ^B activity *B. subtilis*	Boylan et al., 1993
FSL P1-019 (BS PB252 *amyE::P_A-lacZ trpC2*)	Reporter strain for σ^A activity *B. subtilis*	Wise and Price, 1995

10403S *opuCA-gus* strain FSL S1-063 and 192 wells of the otherwise isogenic Δ*sigB opuCA-gus* strain FSL S1-059 was treated with only DMSO, was used as a control. After 3µl of *L. monocytogenes* grown to OD_{600}=0.4 and diluted 1:50, was added to the plate containing the compounds, the plates were sealed and incubated for 18 h at 37°C. To determine bacterial numbers after the incubation period, absorbance (OD_{600}) was measured using a Synergy™ HT Multi-Mode Microplate Reader (BioTek Instruments; Winooski, VT) at approximately 18 h. To prepare the plates for fluorescence measurements, black seals (Perkin Elmer; Waltham, MA) were affixed to the back of the plates after the T=18 h absorbance readings. For the GUS assays, cells were lysed using 5µl of 2x CelLyticB (Sigma; St. Louis, MO and Protease Inhibitor Cocktail (Sigma) mixture (1ml 2x CelLyticB and 0.05ml Protease Inhibitor Cocktail), immediately prior to addition of 4µl of 1.6mg/ml 4-methylumbelliferyl β-D glucuronide hydrate (4-MUG; Sigma) in DMSO. Reactions were incubated in the dark for 1 h at room temperature (~23^{o}C) then reactions were stopped by addition of 0.2M Na_2CO_3 (Sigma). Fluorescence was read using a Wallac 2102 EnVision™ Multilabel Reader (Perkin Elmer) with an excitation wavelength of 355nm and an emission of 460nm.

Statistical analysis of primary screen data

To identify compounds that inhibited σ^B activity without affecting *L. monocytogenes* growth, *opuCA*-directed GUS activity in the presence of each compound was calculated by dividing relative fluorescence units (RFU) by cell density (in OD_{600} units) (RFU/OD, (88)) Statistical analyses were conducted in collaboration with the Broad Institute and performed as previously described (47, 50). Briefly, the median value from the internal DMSO-only wells in a given plate were subtracted from raw values of all 12 to 20 internal DMSO-only wells within the same assay plate. The resulting scores were used to build a distribution across assay plates

between replicates (i.e., replicate plates A and B), which was used to identify outliers within the internal DMSO-only wells (i.e., values that differ from the median by more than 2.57 standard deviations). Values for the DMSO-only wells (after removal of outliers) were used to calculate an average baseline for each plate. Raw values from duplicate plates containing only DMSO inoculated with the wildtype *gus* fusion strain were used to calculate the standard deviation of the baseline value per replicate. The average baseline per plate and standard deviation per replicate, together with raw values from wells with small molecules, were used to calculate Z scores for each small molecule; the Z score represents the deviation of a well with a small molecule from the baseline mean (in standard deviation units). The Z score allows interpretation of the effect of a small molecule on the activity of σ^B. Z scores <-3 standard deviations were considered significant, indicating that a given small molecule inhibits the σ^B. Raw and analyzed data were deposited in ChemBank (86, 94). Z-scores were analyzed using the commercial software package Spotfire DecisionSite Analytics (TIBCO Spotfire; Somerville, MA) to enable 2 dimensional data visualization and identification of positive candidates with high reproducibility. Compounds that generated Z-scores below -3 (based on adjusted RFU/OD data values) were considered to inhibit σ^B activity.

Secondary screen and dose response curve

Forty-one compounds that appeared to inhibit σ^B activity (Z-score $\leq$-3 in both replicates) were selected for secondary cell-based screening using the same format and reporter fusion as described above. These compounds were assessed for their abilities to interfere with σ^B activity and to calculate initial IC_{50} values (i.e., concentration that inhibits 50% of σ^B activity). Each compound was diluted in a series of six 1/5 dilutions of the stock concentration. For example, starting from 19.3mM stock, 8009-2163 was diluted in a series of six 1 to 5 dilutions and pinned into an assay plate. A

total of 14 compounds were assessed for follow-up analyses by "drug developability criteria" including size/molecular weight, hydrogen bonds, structure-based potential toxicity, etc (58).

Small-Molecule Microarray Screens

Small-molecule microarrays (SMMs) were printed on glass slides at the Broad Institute as described previously (12, 13, 22). Two different arrays (each slide printed with 8,500 small-molecule [SM] spots and 1,500 DMSO control spots) were used for our screens. The immobilized SMs included 8,500 compounds from diversity oriented synthesis (DIV06) and 8,500 compounds representing natural products, FDA-approved drug-like compounds, and known bioactive compounds (NPC1) ((22) Chembank.broadinstitute.org). SMM screening (three replicates) was performed as described by Bradner et al. (12). Briefly, His-tagged σ^B was purified from *E. coli* M15 (kindly provided by W. Goebel (8)). Slides were incubated at 4°C with PBST (PBS plus 0.1% Tween 20) containing 1μg/μl of His-tagged σ^B and 1 mg/ml BSA. Slides were washed with PBST buffer 3 times. Slides were incubated in Alexa Fluor 647 labeled anti-His antibody (1:2000 in PBST) for 1 hour at 4°C. Slides were washed 3 times in PBST and 1 time with PBS. Slides were rinsed with deionized water to remove buffer salts and dried by centrifugation at <1000 rpm. Slides were then scanned with an Axon 4000B and analyzed using GenePix® Pro 6 image analysis software. Data analyses included (i) assessment of signal-to-noise ratio (SNR) of the spot feature; (ii) Z-score calculations based on comparison of signals from compound spots compared to signals from DMSO control spots within a slide; and (iii) composite Z-score calculations for data from the three replicates. Spotfire Analytics software was used for 3-dimensional data visualization. Compounds from the DIV 06 library with Z-scores ≥ 0.925 or from the NPC1 library with Z-scores ≥ 1.2 were considered

potential binders. Compounds considered for further analysis were identified by both SMM and HTS.

Phenotypic Profiling and qRT-PCR

The most promising lead compound identified by SMM and HTS (8009-2163) was not commercially available. Therefore, an analog (2-Phenyl-ethenesulfonic acid (4-fluoro-phenyl) amide or T0513-8332, FW 277) was obtained from Enamine Ltd. (Kiev, Ukraine). This compound, T0513-8332 (2-Phenyl-ethenesulfonic acid (4-fluoro-phenyl) amide), designated sigmastatin, has a fluorine substituted for a hydrogen in the original compound. Sigmastatin was dissolved in DMSO to a final concentration of 10mM. The solution was filter sterilized using with a 0.1μm filter compatible with DMSO (OMNIPORE^{TM} Membrane filter, Millipore Corporation, Billerica, MA) which was fitted in a Swinney Stainless 13mm holder for syringe filtration (Millipore Corporation).

Bile Salt Hydrolase (BSH) activity assay

As the *L. monocytogenes bsh* gene, which encodes bile salt hydrolase, is σ^B dependent (23, 45, 72, 96), a BSH activity assay was used to determine the optimal concentration of sigmastatin needed for σ^B inhibition. Four- well multidish plates (26mm x 33mm; Nunc) containing 6ml of either BHI agar or de Man, Rogosa and Sharp (MRS) agar medium (BD Biosciences; San Jose, CA) containing 0.5% (w/v) glycodeoxycholic acid sodium (GDCA) salt (Calbiochem®; San Diego, CA) (21) with either (i) no sigmastatin; or (ii) 96, 193 or 290 μM (5, 10, 15x the 19.3mM stock concentration) of sigmastatin were prepared and allowed to dry overnight. *L. monocytogenes* 10403S and Δ*sigB* were grown in BHI broth to exponential phase, defined as OD_{600}= 0.4, then 4μl of culture was spotted in parallel on the MRS and BHI agars (BHI; Difco, Sparks, MD). The MRS agar plates were incubated in a BD-BBL™ GasPak™ Anaerobic system (BD; Franklin Lakes, NJ) containing an activated

BD-BBL™ GasPak™ Plus anaerobic system envelope with Palladium Catalyst. BHI plates were incubated aerobically. Both sets of plates were incubated for 48 hours at 37°C. The assay was replicated three times.

Cell collection and RNA isolation for qRT-PCR and microarray analyses

L. monocytogenes 10403S and Δ*sigB* strains were grown overnight in 5 ml of BHI broth at 37ºC with shaking (230 rpm), then were sub-cultured twice using a 1% (vol/vol) transfer into 5 ml of pre-warmed BHI. Each time, cells were grown to OD_{600} = 0.4. When the second sub-culture reached OD_{600} = 0.4, cells were treated with a total volume of 76 µl comprised of (i) sigmastatin (to yield final concentrations ranging from 1µuM to 128µM) and/or (ii) DMSO, followed immediately by addition of either 324µl of (i) 5M NaCl (to yield a final concentration of 0.3M NaCl, an osmotic stress that induces σ^B (77)) or (ii) sterile distilled water. Treated cultures were then incubated at 37ºC with shaking (230 rpm) for 10 min. Following incubation, a 2X volume of RNAprotect™ (Qiagen Inc, Valencia, CA.) was added to the treated cultures, mixed and held at room temperature for 10 min. The cells were harvested following centrifugation for 10 min at 5000 X g, supernatant was discarded and tubes containing cell pellets were stored at -80 ºC. RNA was extracted and DNase treated using Ambion RiboPure™-Bacteria Kit (Ambion, Austin, TX) according to the manufacturer's instructions. Total nucleic acid concentrations and purity were assessed using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Rockland, DE). RNA quality was analyzed using the 2100 Bioanalyzer (Agilent Technologies; Santa Clara, CA) and only RNA with an integrity number of ≥8 was used. Each treatment was replicated at least 3 times.

TaqMan qRT-PCR

Transcript levels from the σ^B-dependent genes *opuCA* and *gadA*, as well as from two housekeeping genes, *rpoB* and *gap*, were quantified from the harvested RNA

using TaqMan primers and probes and the ABI Prism® 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) as previously described (46). Data were analyzed using the ABI Prism® 7000 Sequence Detection System (SDS) software (Applied Biosystems) as previously described by Sue et al. 2004. Primers and probes for *gap* and *rpoB* were reported previously (85, 97), respectively. Transcript levels from *opuCA* and *gadA* were $\log_{10}$ transformed and then normalized to the geometric mean of transcript levels from the housekeeping genes *rpoB* and *gap* as previously described (46). Statistical analyses of normalized *opuCA* and *gadA* transcript levels were performed using one-way ANOVA and Tukey's Studentized Range HSD multiple comparison test using SAS® 9.0 (SAS Institute).

Starting with the primary screen concentration (64μM) of sigmastatin, the concentration needed to achieve 50% of maximal inhibition (IC_{50}) was determined by measuring log-normalized transcript levels from *opuCA* collected from cells treated with a series of 1:2 dilutions. These data were analyzed using SigmaPlot® 10.0 (Systat Software Inc.; Evanston, IL) standard curve analysis under the pharmacology function.

Invasion Assay

L. monocytogenes invasion assays using the human colorectal adenocarcinoma epithelial cell line Caco-2 (ATCC HTB-37) were performed as described by Garner et al. (2006) with minor modifications. Briefly, 5.0×10^4 Caco-2 cells were seeded into 24-well plates (Costar, Corning, NY) 48 h prior to infection. *L. monocytogenes* 10403S and Δ*sigB* strains were grown overnight in 5 ml of BHI broth at 37ºC with shaking (230 rpm), then were sub-cultured twice using a 1% (vol/vol) transfer into 5 ml of pre-warmed BHI. Each time, cells were grown to $OD_{600} = 0.4$. When the second sub-culture reached $OD_{600} = 0.4$, cells were treated with a total volume of 76 μl comprised of (i) sigmastatin to yield final concentrations of 64μM (primary screen concentration) or 8μM (lowest concentration with full efficacy according to qRT-

PCR) and/or (ii) DMSO, followed immediately by addition of either 324ul of (i) 5M NaCl or (ii) sterile distilled water. Treated cultures were then incubated at 37ºC with shaking (230 rpm) for 30 min. For infection, the Caco-2 cells were inoculated with approximately 2.0x 10^7 *L. monocytogenes* cells; *L. monocytogenes* numbers used for infection were confirmed by plating on BHI agar. Intracellular *L. monocytogenes* numbers were determined 90 min post infection as previously described (30). Invasion efficiency was calculated as the number of bacteria recovered relative to the lnumber of bacteria used for inoculation (i.e., Log ([CFU/ml recovered] /[CFU/ml inoculated]). Four biological replicates were each performed in triplicate wells. Statistical analysis was performed using one-way ANOVA and Tukey's studentized range (HSD) test, performed in SAS® 9.0 (SAS Institute).

Whole Genome Microarray

cDNA labeling and hydbridization

cDNA labeling was performed as previously described (72) with minor modifications. For cDNA synthesis, 6 µg total RNA was mixed with 3 µg random hexamers and incubated for 10 min at 70°C, then held on ice for 5 min. Superscript III RT, Aminoallyl 2'-Deoxyribonucleoside Triphosphates, dithiothreitol, RNaseOUT, and buffer were added and the reaction was incubated at 42°C for approximately 17 h. The reaction was stopped and purified according to Ollinger et al., 2009 (72). cDNA coupling reactions with Cy™3 or Cy™5 monofunctional fluorescent dyes (GE Healthcare UK Ltd; UK) were performed for 1 h at room temperature. *L. monocytogenes* whole genome microarrays were constructed as previously described (15, 77). Microarray hybridization was performed as previously described (72). Array data were deposited at the Gene Expression Omnibus (GSE16887).

Statistical analysis of microarray data

Raw intensity values for all probes on each array were normalized using pin-tip LOWESS (77) in R v.2.2.1 with the LIMMA package. Signals from two replicate probes on each array were averaged and $\log_2$ transformations were performed after normalization. Differences in transcript levels between strains were determined using a linear model and p values were determined using eBayes. Differences in transcript levels were considered meaningful if they met both adjusted p-values <0.05 (indicating significant expression), fold changes of ≥2 (indicating differential expression) and probe cross-hybridization index (CHI) of >90%. Although lmo0263 was inhibited by sigmastatin, the CHI was 80% and was not included in our assessments. Gene Set Enrichment Analysis (GSEA) (95) was used to identify gene sets that were significantly enriched among genes up or down-regulated in a given mutant strain. GSEA was run on the ranked list of Log Fold Change values obtained from the fitted normalized data in LIMMA with 1000 permutations and exclusion of gene sets with less than 5 or greater than 2000 members. Genes were classified into sets based on the TIGR Comprehensive Microbial Resource (http://cmr.tigr.org) sub-role categories for *L. monocytogenes* EGD-e. False discovery rate q-values less than 0.25 were considered significant (95).

β-galactosidase enzyme assays in *B. subtilis*

B. subtilis strains P1-015 (PB198 *amyE*::*pDH32-ctc trpC2* (11)) and an otherwise isogenic *sigB* mutant P1-017 (PB345 *amyE*::*pDH32-ctc sigBΔ3*::*spc trpC2* (10)) were used as reporter strains for measuring σ^B activity (Table 4.1). The P_{ctc}-*lacZ* reporter fusion was chosen for monitoring σ^B activity as *ctc* transcription has been clearly established as σ^B –dependent (65). A *B. subtilis* strain P1-019 (PB252 *amyE*::P_A-*lacZ trpC2* (104)) was used to assess whether treatment with 64μM of the compound affected the housekeeping sigma factor σ^A in *B. subtilis*. Strains were

grown overnight in 5 ml of buffered Luria Bertani (LB) broth at 37ºC with shaking (230 rpm), then were sub-cultured twice using a 1% (vol/vol) transfer into 5 ml of pre-warmed LB. Each time, cells were grown to OD_{600} = 0.4. When the second sub-culture reached OD_{600} = 0.4, cells were treated with 76µl of (i) sigmastatin and DMSO (final 8 or 64µM) or (ii) DMSO only, followed immediately by addition of 324µl of (i) 5M NaCl (final 0.3M) or (ii) sterile distilled water. Treated cultures were then incubated in the 37ºC shaking incubator for 30 min. OD_{600} was recorded and 0.2ml of the culture was added to a tube containing 2.8ml Z-buffer. 0.02ml toluene was added to permeabilize the cells. Pre-warmed 0.4ml of 4mg/ml ortho-nitrophenyl-β-galactoside (ONPG) was added and the time of addition was noted. The reaction proceeded for 85 minutes, after which, 1ml of 1M sodium carbonate was added to stop the reaction. OD_{420} was read and Miller Units were calculated (106). Statistical anlaysis of β-galactosidase activity was assessed using one-way ANOVA and Tukey's studentized range (HSD) test, performed in SAS® 9.0 (SAS Institute).

RESULTS

A high-throughput cell-based screen identifies promising small molecules that interfere with σ^B activity

A high-throughput cell-based screening assay (HTS) was developed, validated, and used to identify small molecules that inhibit σ^B activity. The premise of the 384-well plate-formatted assay was that a compound that reduced β-glucuronidase activity generated by a reporter fusion between the σ^B–dependent *opuCA* promoter and *gus* (encoding GUS) (96) without affecting *L. monocytogenes* growth (according to plate assay) would be a candidate for further consideration (Chembank Screening Project: SigBInhibition). Based on this primary screen (Figure 4.1), 41 compounds were identified that inhibited σ^B activity (Z score ≤ -3 in both replicates for RFU and

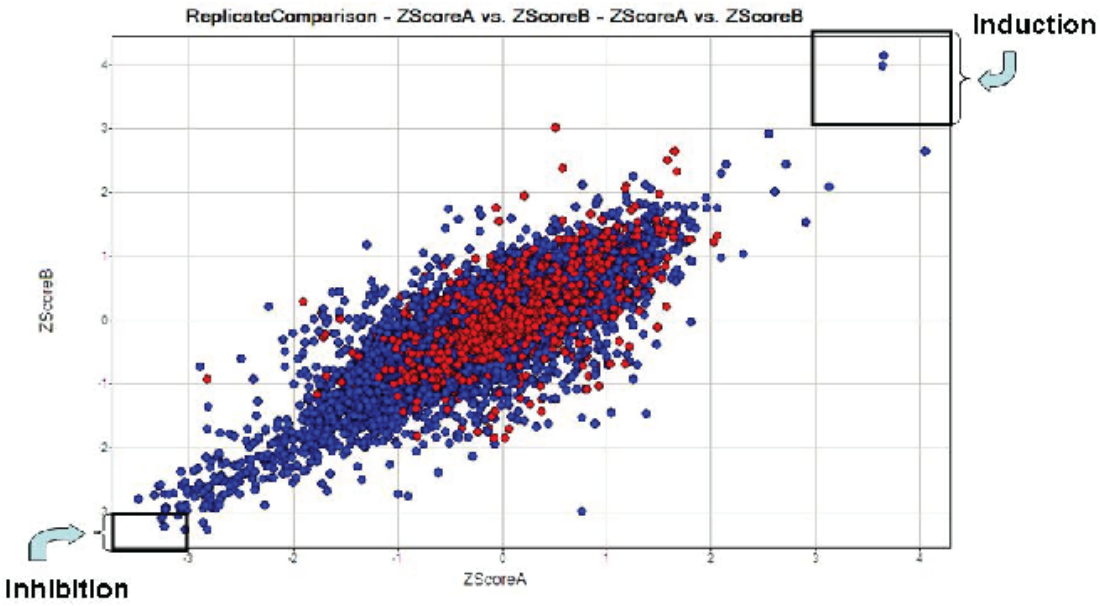


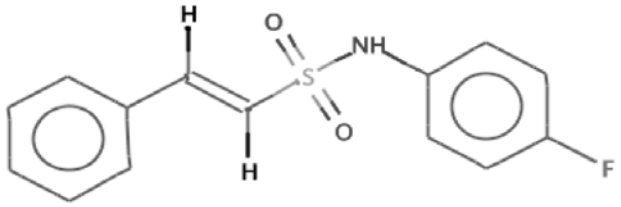
Figure 4.1: Scatterplot of high-throughput screen. Scatterplot of Z-scores calculated from GUS activities (in relative fluorescent units [RFU]) normalized to cell density (in OD_{600} units) for small molecules tested in duplicate in an initial screen. Red dots represent DMSO controls; blue dots are small molecules tested.

RFU/OD and Z score ~0 for OD) and were selected for secondary cell-based screening. Compounds that produced induction were not pursued further.

The secondary screening data obtained from the *opuCA-gus* reporter fusion strain (as described above) in the presence of 6 five-fold dilutions of the initial concentration (e.g. starting from a 64uM final concentration, 8009-2163 was diluted 1 to 5 six times) of each of the 41 compounds, was used to calculate preliminary IC_{50} values (i.e., concentration that inhibits 50% of σ^B activity). 14 compounds inhibited σ^B activity at concentrations lower than initially tested in the primary screen. Based on drug developability criteria (58) and cytotoxicity information in ChemBank (*chembank.broad.harvard.edu*), poor drug candidates were eliminated, leaving three promising compounds that inhibited *L. monocytogenes* σ^B. One particularly effective lead compound, 8009-2163 (IC_{50}= ~15μM), was not commercially available, thus an analog of this compound (T0513-8332) was utilized (Figure 4.2). T0513-8332 (2-Phenyl-ethenesulfonic acid (4-fluoro-phenyl) amide), hereafter designated sigmastatin, has fluorine substituted for the hydrogen in the original compound. While 8009-2163 shows minimal evidence for cytotoxicity, based on cytotoxicity data in ChemBank, sigmastatin shows no evidence for cytotoxicity (*chembank.broad.harvard.edu*).

Identification of σ^B binders using small-molecule microarrays (SMM)

The ability of various small molecules to bind to σ^B was assessed using a SMM screen with His-tagged σ^B. Using replicate Z-scores in Spotfire analytics, 19 high scoring σ^B ligands were identified (see Appendix Figure AF.1 for representative scatterplot). This included 10 compounds from the diversity oriented (DIV06) library and 9 from the natural products and commercial compound (NPC1) library. Several compounds were eliminated because of promiscuity (n=5), non-specific binding (n=5),



sigmastatin

Figure 4.2: Structure of sigmastatin. Chemical structure of σ^B inhibitor sigmastatin (2-Phenyl-ethenesulfonic acid (4-fluoro-phenyl) amide).

or because they were not commercially available (n=6). Three purchasable ligands fit our criteria of lacking promiscuity and non-specificity, but subsequent assays showed they were not effective at inhibiting σ^B activity. Several other compounds were tested but these compounds did not affect σ^B -dependent transcription in phenotypic and genotypic assays. Therefore, while these compounds may bind σ^B, they do not inhibit σ^B activity. Though not among the top 19 binding compounds, sigmastatin was within the top 10% of the strongest σ^B ligands, suggesting the possibility of a direct interaction. These SMM data provide initial evidence that sigmastatin may inhibit σ^B activity by interacting with σ^B

Multiple lines of evidence support σ^B activity inhibition by sigmastatin.

To determine the phenotypic effects of sigmastatin at various concentrations, we qualitatively assessed its effect on the activity of bile salt hydrolase, which is the product of the σ^B-dependent *bsh* and required for survival *in vivo* (98). *L. monocytogenes* treated with sigmastatin at concentrations of 96μM and 193 μM showed no BSH activity (Figure 4.3), although *L. monocytogenes* growth on BHI did not appear to be affected. At 290μM of sigmastatin, *L. monocytogenes* produced no BSH activity and showed complete growth inhibition on BHI (Figure 4.3).

To quantitatively assess the affects of σ^B –driven transcription, quantitative reverse transcriptase PCR (qRT-PCR) was used on mRNA from *L. monocytogenes* that had been exposed to σ^B inducing conditions (i.e., 0.3M NaCl for 10 min) in the presence or absence of various concentrations of sigmastatin. Based on qRT-PCR experiments, there was a ~40-fold reduction in transcript levels for both σ^B -dependent genes *opuCA* and *gadA* following treatment with 64 μM of sigmastatin relative to transcript levels in cells that were not treated with sigmastatin (Figure 4.4a & 4.4b; $p<0.05$, GLM Tukey). Concentration-dependent assessment of the effect of sigmastatin on

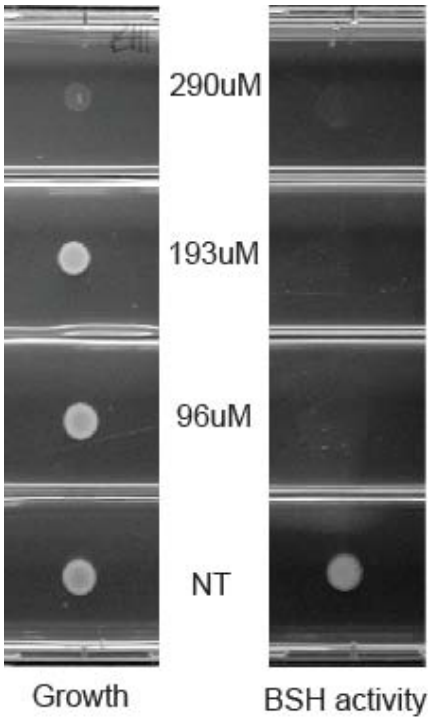
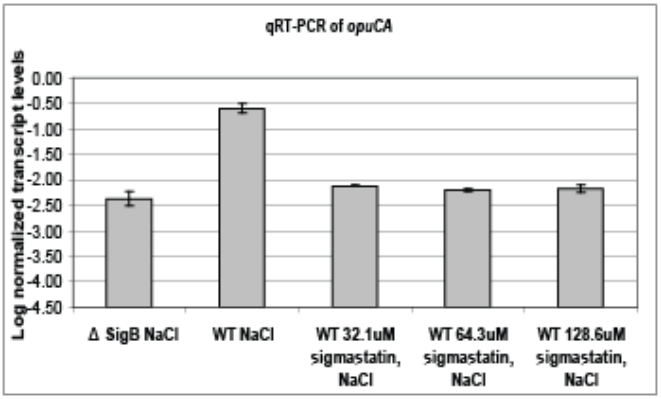
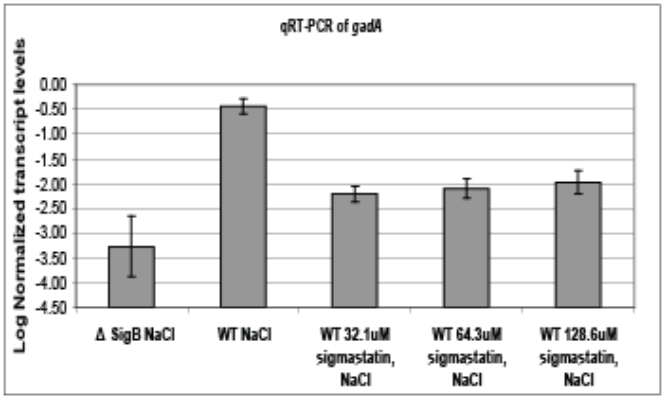


Figure 4.3: Phenotypic agar assay for σ^B-dependent BSH activity in the presence of sigmastatin at various concentrations. Left panel shows *L. monocytogenes* spotted on BHI agar, indicating growth, and the right panel shows *L. monocytogenes* spotted on MRS agar, indicating Bile Salt Hydrolase activity. Each well for both agars contains various concentrations of sigmastatin, from bottom to top: no treatment, 96μM, 193μM, 290μM. *L. monocytogenes* grew on wells of BHI agar containing no treatment, 96μM and 193μM of sigmastatin, while little growth occurred at 290μM. *L. monocytogenes* deconjugated bile salts on MRS containing no sigmastatin, however, BSH was inhibited in all wells containing sigmastatin.



(a)



(b)

Figure 4.4. qRT-PCR graphs illustrating σ^B-dependent *opuCA* and *gadA* transcription. qRT-PCR graphs illustrating normalized log transformed **(a)** *opuCA* and **(b)** *gadA* transcription in *L. monocytogenes* 10403S and Δ*sigB* strains treated with 0.3M NaCl, with or without sigmastatin at various concentrations for 10 min. Values represent mean log normalized transcript levels from at least three independent RNA collections.

transcription showed that transcript levels of *opuCA* in salt-treated 10403S cells, concurrently treated with sigmastatin (ranging from 8μM to 128μM), produced transcript levels which were equivalent to those seen in the Δ*sigB* strain ($p>0.05$). At 4μM, sigmastatin significantly reduced transcription of *opuCA* ($p<0.05$), however, not to levels equivalent to Δ*sigB* strain (data not shown). Using Sigmaplot and log-normalized transcript levels of *opuCA,* the concentration at which half the maximal inhibition (IC_{50}) occurred was determined to be 3.5μM. Importantly, absolute transcript levels for the housekeeping genes *rpoB* and *gap* were similar for 10403S with and without exposure to sigmastatin, indicating that this small molecule specifically inhibits transcription of σ^B-dependent genes and does not affect housekeeping functions. As a consequence of treatment with sigmastatin, there was no further σ^B directed activity in a treated strain as compared to a Δ*sigB* strain. Sigmastatin is effective in that there is not a reduction but elimination of σ^B-dependent activity at levels as low as 8μM.

***L. monocytogenes* whole genome microarray identified 208 genes downregulated by treatment with sigmastatin; 156 of these genes are regulated by σ^B.**

To further characterize the ability of sigmastatin to specifically inhibit σ^B, transcriptional profiling was performed using a *L. monocytogenes* whole-genome microarray. Microarray profiling was performed on four independent RNA collections from log-phase salt stressed *L. monocytogenes* exposed to either 64 μM sigmastatin or to DMSO alone (the solvent used for sigmastatin). Analysis of microarray data (using LIMMA package for R) showed that 208 genes were downregulated and 32 genes were upregulated as a result of treatment with sigmastatin (adj. $p<0.05$ and $FC\geq2$). 156 of the 208 sigmastatin-downregulated genes are positively regulated by σ^B and 7 of the 32 sigmastatin-upregulated genes are negatively regulated by σ^B, based on

previous microarray analysis in 10403S and EGD-e, RNA seq and HMM (Table 4.2, Table 4.3 and 4.4) (35, 45, 71, 72, 77, 98). There are 115 sigmastatin-downregulated genes positively regulated by σ^B in both the *L. monocytogenes* strains 10403S and EGD-e (35, 45, 71, 72, 77, 98). An additional 21 genes are positively regulated by σ^B specifically in the *L. monocytogenes* strain 10403S (45, 71, 72, 77) and another 20 are positively regulated by σ^B specifically in the *L. monocytogenes* strain EGD-e (35, 98). Interestingly, sigmastatin inhibited >90% of genes with an HMM identified σ^B-dependent promoter.

Of the 208 sigmastatin-downregulated genes, 126 were found to be positively regulated by σ^B during infection in the murine intestine (98), including 17 σ^B-dependent genes specific to the intestine (Table 4.4 and 4.5). Among these 17 genes, 9 genes were of unknown or hypothetical function. Three genes lmo0584, lmo0648, lmo0650 were similar to membrane proteins, while lmo0649 and lmo0651 were similar to transcriptional regulators. One gene, lmo1992 was similar to alpha-acetolactate decarboxylase, another, lmo1789, was similar to Nad(P)h Oxidoreductase chain B and a third, lmo0406, was similar to *B. subtilis* YyaH protein.

Results from Gene Set Enrichment Analysis (GSEA, Broad Institute, Cambridge, MA) further supported that sigmastatin extensively inhibited the σ^B regulon, as the known σ^B -regulated genes were significantly enriched among sigmastatin-downregulated genes (False discovery rate (FDR) $q<0.0001$). Note that in GSEA gene sets are considered significant at an FDR $q<0.25$. To assess the effects of sigmastatin on the function of other alternative sigma factors, transcript levels for genes in the σ^H and σ^L regulons were assessed. 14 of the 30 genes identified as σ^H–dependent ($p<0.05$, $FC\geq2$) were significantly and differentially downregulated by sigmastatin (adj. $p<0.05$, $FC\geq2$), however, 12 of those 14 genes are also σ^B -dependent. GSEA showed that the σ^H-only regulon (comprised of genes that are only

Table 4.2: Number of genes differentially expression as a result of treatment with sigmastatin and correlation to σ^B regulation

	208 genes downregulated by sigmastatin	32 genes upregulated by sigmastatin
Positively σ^B regulated	156 [a]	2
Negatively σ^B regulated	6 [b]	7
σ^B-dependent promoters	86 [c]	0

[a] 156 genes includes 4 genes positively regulated by σ^B under some conditions, but negatively regulated by σ^B under other conditions. See supplemental tables 1 and 3.

[b] 6 genes includes 4 genes negatively regulated by σ^B under some conditions, but positively regulated by σ^B under other conditions. See supplemental tables 2 and 3.

[c] σ^B-dependent promoters as determined by *in silico* analysis using Hidden Markov Model (71).

Table 4.3. Comparison of genes downregulated by T0513 and σ^B dependent genes previously identified in 10403S and EGD-e

Gene name	Sigma-statin FC[a]	Sigma-statin adj. p value	σ^B salt FC[b]	σ^B salt adj. p value[b]	σ^B stationary FC[b]	σ^B stationary ad. p value[b]	σ^B dependent promoter[c]	σ^B - dependent in other studies[d]
lmo0019	**-21.3**	**0.0000**	**5.1**	**0.0004**	**3.5**	**0.0010**	+	+
lmo0025	**-10.3**	**0.0000**	1.2	0.0354	1.2	0.1720	.	
lmo0026	**-4.4**	**0.0000**	1.1	0.4304	1.0	0.8397	.	
lmo0036	**-4.9**	**0.0000**	1.6	0.0062	1.1	0.7999	.	
lmo0038	**-3.8**	**0.0000**	1.5	0.0031	1.1	0.3917	.	
lmo0039	**-5.1**	**0.0000**	1.6	0.0103	1.1	0.7672	.	
lmo0043	**-26.0**	**0.0000**	**4.0**	**0.0001**	**3.5**	**0.0003**	+	+
lmo0090	**-2.4**	**0.0000**	1.3	0.0262	1.0	0.8873	.	
lmo0105	**-2.2**	**0.0023**	1.0	0.7338	1.0	0.7668	.	
lmo0129	**-2.4**	**0.0000**	1.1	0.6574	1.1	0.8473	.	
lmo0133	**-4.5**	**0.0000**	**2.4**	**0.0070**	1.2	0.4341	+	+
lmo0134	**-29.6**	**0.0000**	**6.7**	**0.0001**	**6.2**	**0.0001**	+	+
lmo0169	**-11.7**	**0.0000**	**2.8**	**0.0044**	**2.1**	**0.0025**	+	+
lmo0170	**-12.5**	**0.0000**	**4.0**	**0.0003**	1.9	0.1905	+	+
lmo0232	**-2.1**	**0.0000**	**2.5**	**0.0010**	0.8	0.4050	.	
lmo0274	**-3.5**	**0.0000**	1.1	0.8128	1.2	0.2321	+	+
lmo0291	**-5.2**	**0.0000**	**2.0**	**0.0004**	**2.1**	**0.0006**	.	+
lmo0321	**-29.0**	**0.0000**	**3.0**	**0.0006**	**4.3**	**0.0000**	+	+
lmo0326	**-2.1**	**0.0019**	1.0	0.9104	1.0	0.9343	.	
lmo0335	**-3.2**	**0.0000**	1.3	0.0081	1.0	0.9526	.	
lmo0336	**-3.6**	**0.0000**	1.2	0.3953	1.1	0.8672	.	
lmo0337	**-3.0**	**0.0000**	1.3	0.0252	1.1	0.7318	.	
lmo0339	**-3.9**	**0.0000**	1.4	0.0269	1.1	0.6423	.	
lmo0405	**-11.3**	**0.0000**	**2.0**	**0.0012**	**2.1**	**0.0010**	+	+
lmo0406	**-5.0**	**0.0000**	1.5	0.0028	1.6	0.0158	.	+
lmo0407	**-2.2**	**0.0000**	1.3	0.1307	1.5	0.0029	.	+
lmo0408	**-2.6**	**0.0000**	1.2	0.1957	1.5	0.0074	.	+
lmo0433	**-31.3**	**0.0000**	**2.0**	**0.0135**	1.3	0.0608	+	+
lmo0434	**-24.6**	**0.0000**	**2.0**	**0.0124**	1.4	0.0085	+	+
lmo0439	**-14.4**	**0.0000**	1.7	0.0073	1.4	0.1021	+	+
lmo0445	**-16.1**	**0.0000**	**2.5**	**0.0006**	**3.0**	**0.0004**	.	+
lmo0479	**-7.7**	**0.0000**	1.3	0.0362	1.4	0.0852	.	
lmo0515	**-19.9**	**0.0000**	**2.6**	**0.0020**	**2.1**	**0.0116**	+	+
lmo0524	**-4.5**	**0.0000**	1.5	0.4178	1.9	0.0007	.	+
lmo0528	**-2.0**	**0.0000**	1.0	0.9412	1.2	0.1568	.	
lmo0529	**-2.4**	**0.0000**	1.1	0.3424	1.1	0.3850	.	+
lmo0530	**-2.3**	**0.0000**	0.9	0.8041	1.1	0.6027	.	
lmo0531	**-2.4**	**0.0000**	1.1	0.7082	1.1	0.8592	.	
lmo0539	**-21.6**	**0.0000**	**18.8**	**0.0001**	**13.7**	**0.0000**	+	+
lmo0554	**-28.5**	**0.0000**	**4.0**	**0.0004**	**4.4**	**0.0001**	+	+
lmo0555	**-11.2**	**0.0000**	**2.8**	**0.0032**	**4.1**	**0.0003**	+	+
lmo0579	**-3.5**	**0.0000**	1.5	0.0225	1.5	0.0035	.	+
lmo0580	**-3.2**	**0.0000**	**2.2**	**0.0002**	1.6	0.0219	.	+
lmo0584	**-3.0**	**0.0000**	1.2	0.1896	1.4	0.0342	.	+
lmo0589	**-7.6**	**0.0000**	**3.9**	**0.0002**	1.8	0.0018	.	
lmo0590	**-8.2**	**0.0000**	**4.1**	**0.0002**	1.9	0.0047	.	+
lmo0591	**-10.2**	**0.0000**	**5.4**	**0.0008**	1.7	0.0006	.	+
lmo0593	**-3.0**	**0.0000**	1.5	0.0092	**4.3**	**0.0001**	+	+
lmo0596	**-86.4**	**0.0000**	**17.7**	**0.0001**	**12.3**	**0.0002**	+	+
lmo0598	**-2.8**	**0.0005**	1.0	0.8496	1.1	0.7203		

Table 4.3 (Continued)

Gene name	Sigma-statin FC[a]	Sigma-statin adj. p value	σ^B salt FC[b]	σ^B salt adj. p value[b]	σ^B stationary FC[b]	σ^B stationary ad. p value[b]	σ^B dependent promoter[c]	σ^B - dependent in other studies[d]
lmo0602	**-21.9**	**0.0000**	**5.5**	**0.0003**	**3.0**	**0.0007**	+	+
lmo0606	**-8.9**	**0.0000**	1.5	0.0045	1.2	0.1320	.	
lmo0607	**-12.6**	**0.0000**	**2.1**	**0.0011**	1.1	0.4354	.	
lmo0608	**-13.5**	**0.0000**	**3.3**	**0.0001**	0.8	0.1862	.	
lmo0610	**-20.6**	**0.0000**	1.6	0.0015	**2.3**	**0.0006**	+	+
lmo0626	**-2.3**	**0.0000**	1.3	0.0421	1.2	0.2172	.	+
lmo0628	**-27.1**	**0.0000**	**2.2**	**0.0018**	**3.8**	**0.0002**	+	+
lmo0629	**-6.2**	**0.0000**	1.7	0.0126	**2.3**	**0.0002**	+	+
lmo0642	**-3.7**	**0.0000**	1.1	0.5652	1.5	0.4353	.	
lmo0647	**-7.8**	**0.0000**	**4.8**	**0.0005**	**3.6**	**0.0029**	.	+
lmo0648	**-4.2**	**0.0000**	1.6	0.1804	1.6	0.0031	.	+
lmo0649	**-3.8**	**0.0000**	1.3	0.1622	1.4	0.0076	.	+
lmo0650	**-3.4**	**0.0000**	1.3	0.0413	1.2	0.1961	.	+
lmo0651	**-2.1**	**0.0000**	1.4	0.0385	1.0	0.9298	.	+
lmo0653	**-2.2**	**0.0000**	**2.3**	**0.0025**	1.2	0.5446	.	
lmo0654	**-16.3**	**0.0000**	**2.2**	**0.0004**	**2.1**	**0.0027**	+	+
lmo0655	**-12.7**	**0.0000**	**2.5**	**0.0006**	**2.0**	**0.0004**	+	+
lmo0669	**-71.3**	**0.0000**	**34.4**	**0.0001**	**34.4**	**0.0000**	+	+
lmo0670	**-51.4**	**0.0000**	**12.8**	**0.0001**	**8.3**	**0.0001**	+	+
lmo0671	**-2.0**	**0.0000**	1.1	0.7433	1.0	0.9100	.	
lmo0722	**-22.8**	**0.0000**	**4.5**	**0.0005**	**2.3**	**0.0074**	+	+
lmo0759	**-3.6**	**0.0000**	1.3	0.0383	1.3	0.0138	.	+
lmo0760	**-3.6**	**0.0000**	1.3	0.0322	1.4	0.0076	.	+
lmo0761	**-3.3**	**0.0000**	1.2	0.6426	1.0	0.9955	.	+
lmo0781	**-14.8**	**0.0000**	2.9	0.3955	**3.3**	**0.0000**	+	+
lmo0782	**-22.9**	**0.0000**	**10.3**	**0.0001**	**12.6**	**0.0000**	+	+
lmo0783	**-26.9**	**0.0000**	**8.9**	**0.0001**	**10.8**	**0.0000**	+	+
lmo0784	**-24.0**	**0.0000**	**8.5**	**0.0001**	**10.2**	**0.0000**	+	+
lmo0794	**-28.1**	**0.0000**	**2.3**	**0.0016**	**5.5**	**0.0002**	+	+
lmo0796	**-16.1**	**0.0000**	**6.7**	**0.0001**	**3.7**	**0.0000**	+	+
lmo0819	**-4.7**	**0.0000**	1.6	0.0062	1.4	0.0267	.	+
lmo0820	**-2.7**	**0.0000**	1.0	0.8773	1.1	0.8282	.	
lmo0821	**-2.2**	**0.0000**	1.3	0.0085	1.4	0.0105	.	
lmo0869	**-3.0**	**0.0000**	1.2	0.0386	1.5	0.0282	.	+
lmo0880	**-70.7**	**0.0000**	**12.3**	**0.0001**	**13.0**	**0.0002**	+	+
lmo0893	**-6.6**	**0.0000**	**2.2**	**0.0005**	1.5	0.0071	+	+
lmo0894	**-6.9**	**0.0000**	**2.4**	**0.0011**	1.3	0.0473	+	+
lmo0895	**-6.7**	**0.0000**	**5.1**	**0.0001**	**2.1**	**0.0004**	+	+
lmo0896	**-6.9**	**0.0000**	**4.2**	**0.0003**	**2.2**	**0.0003**	+	+
lmo0904	**-2.7**	**0.0000**	1.2	0.0835	1.5	0.0065	.	
lmo0911	**-5.7**	**0.0000**	**3.8**	**0.0004**	**2.7**	**0.0006**	+	+
lmo0913	**-80.0**	**0.0000**	**9.7**	**0.0002**	**5.8**	**0.0001**	+	+
lmo0915	**-6.6**	**0.0000**	1.2	0.3224	0.8	0.1093	.	
lmo0937	**-4.7**	**0.0000**	**3.3**	**0.0090**	**3.8**	**0.0006**	+	+
lmo0953	**-20.8**	**0.0000**	**3.0**	**0.0007**	**3.0**	**0.0001**	+	+
lmo0956	**-5.0**	**0.0000**	**2.7**	**0.0001**	**2.3**	**0.0000**	.	+
lmo0957	**-3.2**	**0.0000**	**2.2**	**0.0003**	1.7	0.0010	.	+
lmo0958	**-2.4**	**0.0000**	**2.0**	**0.0002**	1.5	0.0634	.	+
lmo0994	**-39.8**	**0.0000**	**7.6**	**0.0003**	**7.3**	**0.0000**	+	+
lmo0995	**-35.6**	**0.0000**	**3.9**	**0.0003**	**2.8**	**0.0005**	.	+

Table 4.3 (Continued)

Gene name	Sigma-statin FC[a]	Sigma-statin adj. p value	σ^B salt FC[b]	σ^B salt adj. p value[b]	σ^B stationary FC[b]	σ^B stationary ad. p value[b]	σ^B dependent promoter[c]	σ^B - dependent in other studies[d]
lmo1018	**-2.1**	**0.0000**	0.9	0.1656	1.3	0.0631	.	
lmo1026	**-2.0**	**0.0000**	1.8	0.2523	0.8	0.0637	.	
lmo1027	**-2.0**	**0.0000**	**2.5**	**0.0011**	0.8	0.1705	.	
lmo1139	**-2.1**	**0.0000**	0.8	0.3268	1.2	0.1902	.	+
lmo1140	**-10.8**	**0.0000**	**4.5**	**0.0001**	**5.9**	**0.0000**	.	+
lmo1241	**-22.7**	**0.0000**	**2.3**	**0.0017**	**2.6**	**0.0001**	+	+
lmo1251	**-3.2**	**0.0000**	1.1	0.7622	1.0	0.8895	.	
lmo1261	**-12.0**	**0.0000**	1.9	0.0003	1.8	0.0195	.	+
lmo1266	**-2.0**	**0.0000**	0.5	0.0011	1.3	0.2658	.	
lmo1293	**-4.1**	**0.0000**	**2.2**	**0.0003**	0.3	0.0018	.	+/-
lmo1295	**-4.0**	**0.0000**	**2.5**	**0.0003**	**3.9**	**0.0000**	+	+
lmo1340	**-2.2**	**0.0000**	1.0	0.8446	1.1	0.6705	.	+
lmo1375	**-16.5**	**0.0000**	**2.9**	**0.0006**	**3.4**	**0.0003**	.	+
lmo1421	**-9.0**	**0.0000**	**3.1**	**0.0002**	1.5	0.0410	+	+
lmo1422	**-8.2**	**0.0000**	**2.9**	**0.0012**	1.3	0.2771	.	+
lmo1425	**-26.7**	**0.0000**	**7.0**	**0.0003**	1.8	0.0027	+	+
lmo1426	**-15.8**	**0.0000**	**2.7**	**0.0028**	1.0	0.9582	+	+
lmo1427	**-27.1**	**0.0000**	**3.0**	**0.0004**	**2.0**	**0.0011**	+	+
lmo1428	**-49.9**	**0.0000**	**10.1**	**0.0004**	**2.9**	**0.0000**	+	+
lmo1432	**-2.3**	**0.0000**	1.8	0.0019	**2.1**	**0.0002**	.	+
lmo1433	**-35.6**	**0.0000**	**2.7**	**0.0014**	**2.6**	**0.0004**	+	+
lmo1452	**-3.2**	**0.0000**	1.0	0.8390	1.0	0.8428	.	
lmo1453	**-3.2**	**0.0000**	1.1	0.4123	1.2	0.1549	.	+
lmo1454	**-3.7**	**0.0000**	1.1	0.6346	1.0	0.8592	.	+
lmo1526	**-9.1**	**0.0000**	**2.6**	**0.0003**	1.9	0.0459	+	+
lmo1538	**-3.2**	**0.0000**	1.4	0.2131	0.3	0.0005	.	+/-
lmo1539	**-3.7**	**0.0000**	1.8	0.0453	0.2	0.0001	.	+/-
lmo1580	**-4.1**	**0.0000**	1.9	0.0039	**2.6**	**0.0000**	.	+
lmo1601	**-3.3**	**0.0000**	**2.5**	**0.0004**	**4.2**	**0.0000**	+	+
lmo1602	**-2.7**	**0.0001**	**3.6**	**0.0001**	**5.1**	**0.0000**	+	+
lmo1605	**-2.0**	**0.0000**	1.3	0.0478	1.7	0.0458	.	+
lmo1606	**-2.9**	**0.0000**	1.2	0.1152	**2.8**	**0.0005**	.	+
lmo1666	**-6.2**	**0.0000**	1.8	0.0125	1.5	0.0152	.	
lmo1694	**-28.9**	**0.0000**	**3.4**	**0.0002**	**2.6**	**0.0002**	+	+
lmo1698	**-2.9**	**0.0000**	1.5	0.0862	**2.0**	**0.0004**	+	+
lmo1704	**-2.3**	**0.0000**	1.4	0.0110	1.8	0.0005	.	+
lmo1789	**-3.7**	**0.0000**	1.3	0.0341	1.4	0.0096	.	+
lmo1790	**-2.0**	**0.0000**	1.8	0.0315	1.5	0.0199	.	+
lmo1830	**-8.8**	**0.0000**	**2.0**	**0.0078**	**2.4**	**0.0007**	+	+
lmo1883	**-5.4**	**0.0000**	**2.5**	**0.0013**	**12.0**	**0.0000**	+	+
lmo1912	**-2.4**	**0.0000**	1.0	0.9728	1.1	0.4354	.	
lmo1913	**-2.2**	**0.0000**	1.0	0.8086	0.9	0.6772	.	
lmo1929	**-2.0**	**0.0000**	0.9	0.5359	1.5	0.0495	.	+
lmo1930	**-2.1**	**0.0000**	1.2	0.1519	1.5	0.0341	.	
lmo1992	**-3.0**	**0.0000**	1.9	0.0013	0.6	0.2004	.	+/-
lmo2006	**-2.1**	**0.0000**	0.7	0.0171	0.5	0.0036	.	-
lmo2066	**-2.2**	**0.0000**	1.2	0.1757	0.9	0.6796	.	-
lmo2067	**-37.5**	**0.0000**	**3.2**	**0.0004**	**4.4**	**0.0004**	+	+
lmo2085	**-21.1**	**0.0000**	**5.0**	**0.0002**	**11.1**	**0.0000**	+	+
lmo2092	**-2.4**	**0.0000**	1.2	0.1013	1.0	0.8592	.	

Table 4.3 (Continued)

Gene name	Sigma-statin FC[a]	Sigma-statin adj. p value	σ^B salt FC[b]	σ^B salt adj. p value[b]	σ^B stationary FC[b]	σ^B stationary ad. p value[b]	σ^B dependent promoter[c]	σ^B - dependent in other studies[d]
lmo2130	**-3.8**	**0.0000**	1.3	0.0643	2.5	0.0892	.	+
lmo2131	**-3.0**	**0.0000**	1.1	0.2793	1.0	0.9253	.	
lmo2132	**-9.4**	**0.0000**	1.8	0.0133	**3.8**	**0.0005**	+	+
lmo2157	**-40.4**	**0.0000**	**13.9**	**0.0001**	**11.6**	**0.0000**	+	+
lmo2158	**-22.9**	**0.0000**	**17.8**	**0.0429**	**15.6**	**0.0000**	+	+
lmo2173	**-2.6**	**0.0000**	1.2	0.0597	0.9	0.3349	.	
lmo2174	**-6.7**	**0.0000**	1.2	0.0785	1.2	0.1434	.	+
lmo2191	**-2.5**	**0.0000**	**3.5**	**0.0001**	**2.7**	**0.0001**	.	+
lmo2205	**-3.8**	**0.0000**	**4.3**	**0.0001**	**2.2**	**0.0020**	.	+
lmo2213	**-55.2**	**0.0000**	**14.1**	**0.0001**	**11.6**	**0.0000**	+	+
lmo2230	**-54.7**	**0.0000**	**33.9**	**0.0001**	**21.8**	**0.0001**	+	+
lmo2231	**-21.0**	**0.0000**	**2.6**	**0.0106**	**2.3**	**0.0004**	.	+
lmo2232	**-2.6**	**0.0000**	**4.9**	**0.0025**	1.6	0.0104	.	
lmo2269	**-11.9**	**0.0000**	**2.5**	**0.0092**	**5.7**	**0.0000**	+	+
lmo2281	**-2.5**	**0.0000**	1.1	0.1657	1.2	0.1188	.	
lmo2283	**-2.2**	**0.0000**	1.1	0.3049	1.0	0.8299	.	
lmo2356	**-3.8**	**0.0000**	1.2	0.0938	1.1	0.8214	.	
lmo2357	**-2.3**	**0.0000**	1.3	0.1550	1.3	0.0589	.	
lmo2358	**-3.0**	**0.0000**	1.2	0.0700	1.1	0.5653	.	
lmo2375	**-2.1**	**0.0000**	0.8	0.1706	1.1	0.2510	.	
lmo2386	**-4.4**	**0.0000**	**2.3**	**0.0017**	1.6	0.0012	.	+
lmo2387	**-30.1**	**0.0000**	1.2	0.2264	**2.1**	**0.0019**	+	+
lmo2391	**-26.4**	**0.0000**	**5.7**	**0.0003**	**7.5**	**0.0000**	+	+
lmo2398	**-8.7**	**0.0000**	**7.0**	**0.0001**	**2.3**	**0.0006**	.	+
lmo2399	**-4.3**	**0.0000**	1.9	0.0136	1.3	0.1394	.	+
lmo2400	**-4.4**	**0.0000**	1.4	0.0179	1.3	0.1273	.	
lmo2434	**-21.7**	**0.0000**	**4.1**	**0.0023**	**3.4**	**0.0001**	+	+
lmo2436	**-2.0**	**0.0000**	1.1	0.4100	1.3	0.0918	.	
lmo2454	**-3.3**	**0.0000**	1.6	0.0377	**4.4**	**0.0000**	+	+
lmo2463	**-13.0**	**0.0000**	1.7	0.0029	**2.3**	**0.0001**	+	+
lmo2471	**-2.1**	**0.0000**	**2.2**	**0.0001**	1.0	0.9515	.	
lmo2484	**-2.2**	**0.0008**	**6.2**	**0.0002**	**5.2**	**0.0000**	.	+
lmo2485	**-2.2**	**0.0004**	**4.4**	**0.0002**	**3.7**	**0.0000**	.	+
lmo2494	**-12.4**	**0.0000**	**2.4**	**0.0006**	**2.7**	**0.0001**	.	+
lmo2570	**-3.5**	**0.0010**	**3.1**	**0.0016**	1.9	0.0019	+	+
lmo2571	**-32.9**	**0.0000**	**6.2**	**0.0001**	**4.0**	**0.0000**	+	+
lmo2572	**-27.3**	**0.0000**	**4.4**	**0.0005**	**3.5**	**0.0000**	+	+
lmo2573	**-36.1**	**0.0000**	**4.9**	**0.0004**	**4.0**	**0.0000**	+	+
lmo2602	**-10.8**	**0.0000**	1.4	0.0393	**2.2**	**0.0004**	+	+
lmo2603	**-5.6**	**0.0000**	**2.9**	**0.0026**	**3.8**	**0.0004**	+	+
lmo2670	**-6.0**	**0.0000**	**2.3**	**0.0031**	1.9	0.0015	+	+
lmo2671	**-7.9**	**0.0000**	**2.8**	**0.0002**	**2.8**	**0.0000**	+	+
lmo2672	**-9.5**	**0.0000**	1.9	0.0137	1.7	0.0738	+	+
lmo2673	**-46.3**	**0.0000**	**7.4**	**0.0003**	**13.2**	**0.0000**	+	+
lmo2674	**-3.5**	**0.0000**	1.8	0.0033	**2.7**	**0.0000**	+	+
lmo2685	**-2.2**	**0.0000**	0.9	0.4081	1.2	0.1862	.	
lmo2695	**-20.8**	**0.0000**	**7.0**	**0.0001**	1.3	0.2896	.	+
lmo2696	**-20.6**	**0.0000**	**6.5**	**0.0001**	1.5	0.0469	.	+
lmo2697	**-6.6**	**0.0000**	1.9	0.0146	1.9	0.0147	.	+
lmo2724	**-12.6**	**0.0000**	**3.7**	**0.0010**	1.8	0.0033	+	+

Table 4.3 (Continued)

Gene name	Sigma-statin FC[a]	Sigma-statin adj. p value	σ^B salt FC[b]	σ^B salt adj. p value[b]	σ^B stationary FC[b]	σ^B stationary ad. p value[b]	σ^B dependent promoter[c]	σ^B - dependent in other studies[d]
lmo2733	**-3.2**	**0.0000**	1.0	0.8786	1.1	0.2455	+	+
lmo2734	**-2.1**	**0.0007**	1.2	0.0718	1.2	0.2295	.	
lmo2735	**-2.0**	**0.0023**	1.1	0.2246	1.2	0.1684	.	
lmo2739	**-5.0**	**0.0000**	**2.2**	**0.0007**	1.3	0.0715	.	
lmo2740	**-4.6**	**0.0000**	1.9	0.0008	1.2	0.1449	.	
lmo2741	**-4.6**	**0.0000**	1.8	0.0057	1.2	0.0869	.	
lmo2748	**-67.5**	**0.0000**	**15.6**	**0.0002**	**8.9**	**0.0000**	+	+
LMOinlD	**-7.4**	**0.0000**	1.2	0.2104	1.1	0.6806	+	+

[a] FC indicates fold change

[b] This work was reported in Raengpradub et al., 2008.

[c] σ^B -dependent promoter was determined by HMM in Oliver et al., 2009.

[d] Other studies: Hain et al., 2008, Kazmierczak et al., 2003, Oliver et al., 2009, Ollinger et al., 2009, Toledo-Arana et al., 2009.

Items listed in **bold** are significantly and differentially expressed; "+" indicates positively regulated in other studies and "-" indicates negatively regulated in other studies. "+/-" indicates positively and negatively regulated in other studies

Table 4.4. Comparison of genes upregulated by sigmastatin and σ^B dependent genes previously identified in 10403S and EGD-e

Gene name	Sigma-statin FC[a]	Sigma-statin adj. p value	σ^B salt FC[b]	σ^B salt adj. p value[b]	σ^B stationary FC[b]	σ^B stationary adj. p value[b]	σ^B dependent promoter[c]	σ^B dependent in other studies[d]
lmo0194	**2.1**	**0.0000**	1.0	0.7701	0.9	0.5751	.	
lmo0195	**2.5**	**0.0001**	0.9	0.1975	0.8	0.1647	.	
lmo0560	**2.4**	**0.0000**	0.7	0.0872	**0.4**	**0.0000**	.	
lmo0604	**2.6**	**0.0000**	1.0	0.8953	0.9	0.1543	.	
lmo0678	**2.3**	**0.0008**	0.9	0.5684	0.9	0.5568	.	
lmo0679	**4.0**	**0.0000**	0.5	0.0008	0.7	0.0897	.	
lmo0680	**4.7**	**0.0000**	0.8	0.0399	0.7	0.0432	.	
lmo0681	**3.3**	**0.0000**	0.6	0.0019	0.9	0.4655	.	
lmo0685	**2.1**	**0.0002**	0.6	0.0315	0.7	0.0179	.	
lmo0686	**2.0**	**0.0000**	0.7	0.3102	1.0	0.9515	.	
lmo0955	**2.0**	**0.0001**	0.9	0.6439	1.2	0.1147	.	
lmo0971	**2.1**	**0.0000**	0.5	0.0015	0.8	0.1608	.	
lmo0973	**2.2**	**0.0000**	0.7	0.0121	0.9	0.8428	.	
lmo0974	**2.6**	**0.0000**	**0.4**	**0.0010**	1.0	0.9231	.	
lmo1440	**2.0**	**0.0000**	0.8	0.1900	1.3	0.2283	.	-
lmo1518	**2.7**	**0.0000**	0.6	0.0004	1.0	0.7859	.	
lmo1637	**2.2**	**0.0000**	1.1	0.7880	1.3	0.1219	.	+
lmo1699	**4.7**	**0.0000**	**0.2**	**0.0001**	0.8	0.3118	.	
lmo1700	**5.7**	**0.0000**	**0.2**	**0.0001**	0.9	0.2974	.	
lmo1919	**2.7**	**0.0000**	1.6	0.0683	1.0	0.9092	.	
lmo2114	**3.7**	**0.0000**	**0.3**	**0.0001**	1.0	0.9829	.	
lmo2115	**3.7**	**0.0000**	0.7	0.0419	0.9	0.7332	.	
lmo2150	**2.9**	**0.0000**	0.5	0.0011	0.8	0.1888	.	
lmo2156	**3.0**	**0.0000**	0.8	0.1686	1.0	0.9706	.	
lmo2177	**3.0**	**0.0000**	1.2	0.0860	0.9	0.2393	.	
lmo2219	**2.3**	**0.0000**	**0.5**	**0.0007**	1.0	0.7667	.	
lmo2439	**2.7**	**0.0000**	0.7	0.0465	1.2	0.5422	.	
lmo2567	**2.4**	**0.0000**	1.0	0.9800	1.1	0.3660	.	
lmo2568	**2.0**	**0.0000**	1.0	0.9544	1.1	0.8473	.	+
lmo2687	**3.5**	**0.0000**	1.1	0.6130	0.9	0.4532	.	
lmo2688	**2.7**	**0.0000**	1.4	0.1067	1.0	0.8594	.	
lmo2689	**3.0**	**0.0000**	0.9	0.7075	1.0	0.8351	.	

[a] FC indicates fold change

[b] This work was reported in Raengpradub et al., 2008.

[c] σ^B -dependent promoter was determined by HMM in Oliver et al., 2009.

[d] Other studies: Hain et al., 2008, Kazmierczak et al., 2003, Ollinger et al., 2009, Toledo-Arana et al., 2009.

Items listed in **bold** are significantly and differentially expressed; "+" indicates positively regulated in other studies and "-" indicates negatively regulated in other studies. "+/-" indicates positively and negatively regulated in other studies

Table 4.5: Positively regulated σ^B dependent genes previously identified in *L. monocytogenes* 10403S and EGD-e

Gene name	Gene symbol	sigmastatin	Raengpradub et al. 2008	Kazmierczak et al. 2003	Ollinger et al. 2009	Oliver et al. 2009	Toledo-Arana et al. 2009	Hain et al. 2008	σ^B dependent promoter[a]	σ^B OVERALL[b]
inlD	inlD	-	+	+					+	+
lmo0013	qoxA			+			+			+
lmo0019	lmo0019	-	+		+	+	+	+	+	+
lmo0043	lmo0043	-	+		+	+	+	+	+	+
lmo0045	ssb							+		+
lmo0100	lmo0100						+			+
lmo0122	lmo0122					+				+
lmo0133	lmo0133	-	+		+	+	+		+	+
lmo0134	lmo0134	-	+		+	+	+	+	+	+
lmo0137	lmo0137		+							+
lmo0169	lmo0169	-	+		+	+	+	+	+	+
lmo0170	lmo0170	-	+		+	+	+	+	+	+
lmo0200	prfA				-		+			+/-
lmo0202	hly		-				+			+/-
lmo0205	plcB						+			+
lmo0210	ldh		+		+					+
lmo0211	ctc		+	+						+
lmo0231	lmo0231		+		+					+
lmo0232	clpC	-	+							+
lmo0263[c]	lmo0263		+		+	+	+	+	+	+
lmo0265	lmo0265				+	+	+	+	+	+
lmo0274	lmo0274	-				+	+		+	+
lmo0291	lmo0291	-	+		+					+
lmo0292	lmo0292				+		+			+
lmo0293	lmo0293		+							+
lmo0314	lmo0314		+							+
lmo0321	lmo0321	-	+		+	+	+		+	+
lmo0341	lmo0341		+							+
lmo0342	lmo0342		+							+
lmo0343	lmo0343		+							+
lmo0344	lmo0344		+							+
lmo0345	lmo0345		+							+
lmo0346	lmo0346		+							+
lmo0347	lmo0347		+							+
lmo0348	lmo0348		+							+

Table 4.5 (continued)

Gene name	Gene symbol	sigmastatin	Raengpradub et al. 2008	Kazmierczak et al. 2003	Ollinger et al. 2009	Oliver et al. 2009	Toledo-Arana et al. 2009	Hain et al. 2008	σ^B dependent promoter[a]	σ^B OVERALL[b]
lmo0351	lmo0351				+					+
lmo0353	lmo0353		+							+
lmo0372	lmo0372					+			+	+
lmo0373	lmo0373								+	+
lmo0374	lmo0374								+	+
lmo0386	lmo0386		+							+
lmo0398	lmo0398		+							+
lmo0399	lmo0399		+							+
lmo0400	lmo0400		+							+
lmo0401	lmo0401		+							+
lmo0402	lmo0402		+							+
lmo0403	lmo0403						+			+
lmo0405	lmo0405	-	+	+	+	+	+	+	+	+
lmo0406	lmo0406	-					+			+
lmo0407	lmo0407	-					+			+
lmo0408	lmo0408	-			+		+			+
lmo0433	inlA	-	+	+		+	+		+	+
lmo0434	inlB	-	+	+		+	+		+	+
lmo0438	lmo0438			+						+
lmo0439	lmo0439	-			+	+	+	+	+	+
lmo0445	lmo0445	-	+		+	+	+			+
lmo0495	lmo0495		+							+
lmo0514	lmo0514						+			+
lmo0515	lmo0515	-	+		+	+	+	+	+	+
lmo0524	lmo0524	-		+	+		+			+
lmo0529	lmo0529	-						+		+
lmo0539	lmo0539	-	+		+	+	+	+	+	+
lmo0554	lmo0554	-	+		+	+	+	+	+	+
lmo0555	lmo0555	-	+		+	+	+	+	+	+
lmo0579	lmo0579	-			+		+			+
lmo0580	lmo0580	-	+		+		+	+		+
lmo0582	iap				+			-		+/-
lmo0584	lmo0584	-					+			+
lmo0589	lmo0589	-	+							+

Table 4.5 (continued)

Gene name	Gene symbol	sigmastatin	Raengpradub et al. 2008	Kazmierczak et al. 2003	Ollinger et al. 2009	Oliver et al. 2009	Toledo-Arana et al. 2009	Hain et al. 2008	σ^B dependent promoter[a]	σ^B OVERALL[b]
lmo0590	lmo0590	-	+					+		+
lmo0591	lmo0591	-	+					+		+
lmo0592	lmo0592		+							+
lmo0593	lmo0593	-	+	+	+	+	+	+	+	+
lmo0596	lmo0596	-	+		+	+	+	+	+	+
lmo0602	lmo0602	-	+		+	+	+	+	+	+
lmo0607	lmo0607	-	+							+
lmo0608	lmo0608	-	+							+
lmo0610	lmo0610	-	+		+	+	+	+	+	+
lmo0626	lmo0626	-					+			+
lmo0628	lmo0628	-	+		+	+	+		+	+
lmo0629	lmo0629	-	+		+	+	+		+	+
lmo0647	lmo0647	-	+				+	+		+
lmo0648	lmo0648	-					+			+
lmo0649	lmo0649	-					+			+
lmo0650	lmo0650	-					+			+
lmo0651	lmo0651	-					+			+
lmo0653	lmo0653	-	+							+
lmo0654	lmo0654	-	+		+	+	+		+	+
lmo0655	lmo0655	-	+		+	+	+		+	+
lmo0669	lmo0669	-	+	+	+	+	+	+	+	+
lmo0670	lmo0670	-	+		+	+	+	+	+	+
lmo0676	lmo0676							+		+
lmo0722	lmo0722	-	+		+	+	+	+	+	+
lmo0723	lmo0723						+			+
lmo0724	lmo0724						+			+
lmo0735	lmo0735							+		+
lmo0736	lmo0736							+		+
lmo0737	lmo0737							+		+
lmo0738	lmo0738							+		+
lmo0739	lmo0739							+		+
lmo0759	lmo0759	-					+			+
lmo0760	lmo0760	-					+			+
lmo0761	lmo0761	-					+			+

Table 4.5 (continued)

Gene name	Gene symbol	sigmastatin	Raengpradub et al. 2008	Kazmierczak et al. 2003	Ollinger et al. 2009	Oliver et al. 2009	Toledo-Arana et al. 2009	Hain et al. 2008	σ^B dependent promoter[a]	σ^B OVERALL[b]
lmo0781	lmo0781	-	+		+	+	+	+	+	+
lmo0782	lmo0782	-	+		+	+	+	+	+	+
lmo0783	lmo0783	-	+		+	+	+	+	+	+
lmo0784	lmo0784	-	+	+	+	+	+	+	+	+
lmo0788	lmo0788		+							+
lmo0794	lmo0794	-	+	+	+	+	+	+	+	+
lmo0796	lmo0796	-	+		+	+	+	+	+	+
lmo0819	lmo0819	-				+		+		+
lmo0830	fbp		+							+
lmo0869	lmo0869	-						+		+
lmo0870	lmo0870						+			+
lmo0880	lmo0880	-	+	+	+	+	+	+	+	+
lmo0893	rsbV	-	+	+	+				+	+
lmo0894	rsbW	-	+	+	+				+	+
lmo0895	sigB	-	+	+	+		+	+	+	+
lmo0896	rsbX	-	+	+	+				+	+
lmo0900	lmo0900						+			+
lmo0911	lmo0911	-	+	+	+	+	+	+	+	+
lmo0913	lmo0913	-	+		+	+	+	+	+	+
lmo0929	lmo0929						+			+
lmo0937	lmo0937	-	+		+	+	+		+	+
lmo0943	fri						+			+
lmo0944	lmo0944						+			+
lmo0953	lmo0953	-	+		+	+	+	+	+	+
lmo0956	lmo0956	-	+	+	+		+	+		+
lmo0957	lmo0957	-	+		+		+	+		+
lmo0958	lmo0958	-	+		+		+			+
lmo0994	lmo0994	-	+	+	+	+	+	+	+	+
lmo0995	lmo0995	-	+		+		+			+
lmo0996	lmo0996		+							+
lmo0997	clpE				-			+		+/-
lmo1001	lmo1001						+			+
lmo1014	gbuA		+							+
lmo1027	lmo1027	-	+							+

Table 4.5 (continued)

Gene name	Gene symbol	sigmastatin	Raengpradub et al. 2008	Kazmierczak et al. 2003	Ollinger et al. 2009	Oliver et al. 2009	Toledo-Arana et al. 2009	Hain et al. 2008	σ^B dependent promoter[a]	σ^B OVERALL[b]
lmo1052	pdhA			+						+
lmo1053	pdhB		+		-					+/-
lmo1055	pdhD							+		+
lmo1068	lmo1068						+	+		+
lmo1113	lmo1113						+			+
lmo1114	lmo1114						+			+
lmo1119	lmo1119						+			+
lmo1134	lmo1134						+			+
lmo1139	lmo1139	-					+			+
lmo1140	lmo1140	-	+		+	+	+	+		+
lmo1154	lmo1154						+			+
lmo1159	lmo1159						+			+
lmo1168	ackA2							+		+
lmo1241	lmo1241	-	+		+	+	+	+	+	+
lmo1245	lmo1245						+			+
lmo1256	lmo1256						+			+
lmo1261	lmo1261	-			+		+	+		+
lmo1291	lmo1291		+							+
lmo1293	glpD	-	+/-		+		+			+/-
lmo1295	lmo1295	-	+		+	+		+	+	+
lmo1340	lmo1340	-			+		+			+
lmo1348	lmo1348		+							+
lmo1375	lmo1375	-	+		+	+	+	+		+
lmo1376	lmo1376				+					+
lmo1387	lmo1387		+							+
lmo1397	cinA				+					+
lmo1421	lmo1421	-	+	+		+	+	+	+	+
lmo1422	lmo1422	-	+				+	+		+
lmo1425	opuCD	-	+	+		+	+	+	+	+
lmo1426	opuCC	-	+	+		+	+	+	+	+
lmo1427	opuCB	-	+	+			+	+	+	+
lmo1428	opuCA	-	+	+			+	+	+	+
lmo1432	lmo1432	-	+		+		+			+
lmo1433	lmo1433	-	+	+	+	+	+	+	+	+

Table 4.5 (continued)

Gene name	Gene symbol	sigmastatin	Raengpradub et al. 2008	Kazmierczak et al. 2003	Ollinger et al. 2009	Oliver et al. 2010	Toledo-Arana et al. 2009	Hain et al. 2008	σ^B dependent promoter[a]	σ^B OVERALL[b]
lmo1434	lmo1434				+					+
lmo1435	lmo1435				+					+
lmo1453	lmo1453	-			+					+
lmo1454	rpoD	-			+					+
lmo1475	hrcA		+							+
lmo1526	lmo1526	-	+		+	+	+	+	+	+
lmo1538	lmo1538	-	-		+		+			+/-
lmo1539	lmo1539	-	-	+			+			+/-
lmo1580	lmo1580	-	+	+				+		+
lmo1601	lmo1601	-	+		+		+	+	+	+
lmo1602	lmo1602	-	+	+	+	+	+	+	+	+
lmo1605	murC	-			+					+
lmo1606	lmo1606	-	+	+	+	+	+	+		+
lmo1637	lmo1637	+						+		+
lmo1672	menE		+							+
lmo1684	lmo1684		+		-					+/-
lmo1694	lmo1694	-	+	+	+	+	+	+	+	+
lmo1698	lmo1698	-	+	+	+	+			+	+
lmo1704	lmo1704	-					+			+
lmo1788	lmo1788						+			+
lmo1789	lmo1789	-					+			+
lmo1790	lmo1790	-					+			+
lmo1830	lmo1830	-	+		+	+	+	+	+	+
lmo1848	lmo1848		+					-		+/-
lmo1849	lmo1849		+/-					-		+/-
lmo1866	lmo1866					+				+
lmo1873	lmo1873		+							+
lmo1883	lmo1883	-	+	+	+	+	+	+	+	+
lmo1929	ndk	-			+		+			+
lmo1932	lmo1932						+			+
lmo1933	lmo1933		+	+	+					+
lmo1934	hup				+					+
lmo1966	lmo1966						+			+
lmo1992	lmo1992	-			-		+			+/-

Table 4.5 (continued)

Gene name	Gene symbol	sigmastatin	Raengpradub et al. 2008	Kazmierczak et al. 2003	Ollinger et al. 2009	Oliver et al. 2010	Toledo-Arana et al. 2009	Hain et al. 2008	σ^B dependent promoter[a]	σ^B OVERALL[b]
lmo1998	lmo1998						+			+
lmo1999	lmo1999						+			+
lmo2000	lmo2000						+			+
lmo2001	lmo2001						+			+
lmo2002	lmo2002						+			+
lmo2003	lmo2003					+	+			+
lmo2004	lmo2004						+			+
lmo2030	lmo2030				+					+
lmo2031	lmo2031				+					+
lmo2041	lmo2041				+					+
lmo2057	ctaB		+							+
lmo2064	lmo2064		+							+
lmo2067	lmo2067	-	+	+	+	+	+	+	+	+
lmo2085	lmo2085	-	+	+	+	+	+	+	+	+
lmo2130	lmo2130	-			+	+				+
lmo2132	lmo2132	-	+		+	+	+		+	+
lmo2153	lmo2153						+			+
lmo2157	sepA	-	+	+	+	+	+	+	+	+
lmo2158	lmo2158	-	+		+	+		+	+	+
lmo2159	lmo2159		+							+
lmo2160	lmo2160		+							+
lmo2161	lmo2161		+/-							+/-
lmo2163	lmo2163		+							+
lmo2169	lmo2169		+							+
lmo2174	lmo2174	-					+			+
lmo2175	lmo2175				-			+		+/-
lmo2191	lmo2191	-	+		+		+			+
lmo2205	lmo2205	-	+	+	+			+		+
lmo2213	lmo2213	-	+		+	+	+		+	+
lmo2230	lmo2230	-	+	+	+	+	+	+	+	+
lmo2231	lmo2231	-	+		+	+	+	+		+
lmo2232	lmo2232	-	+							+
lmo2269	lmo2269	-	+	+	+	+	+		+	+
lmo2271	lmo2271						+			+

Table 4.5 (continued)

Gene name	Gene symbol	sigmastatin	Raengpradub et al. 2008	Kazmierczak et al. 2003	Ollinger et al. 2009	Oliver et al. 2010	Toledo-Arana et al. 2009	Hain et al. 2008	σ^B dependent promoter[a]	σ^B OVERALL[b]
lmo2289	lmo2289						+			+
lmo2290	lmo2290		+							+
lmo2352	lmo2352		+							+
lmo2386	lmo2386	-	+	+	+		+	+		+
lmo2387	lmo2387	-	+		+	+	+	+	+	+
lmo2389	lmo2389			+						+
lmo2391	lmo2391	-	+		+	+	+	+	+	+
lmo2398	ltrC	-	+	+	+	+	+	+		+
lmo2399	lmo2399	-		+						+
lmo2434	lmo2434	-	+	+		+	+		+	+
lmo2437	lmo2437		+							+
lmo2454	lmo2454	-	+		+	+	+		+	+
lmo2456	pgm		+					-		+/-
lmo2463	lmo2463	-	+	+	+	+	+	+	+	+
lmo2471	lmo2471	-	+							+
lmo2484	lmo2484	-	+		+	+	+	+		+
lmo2485	lmo2485	-	+	+	+	+	+	+		+
lmo2494	lmo2494	-	+		+	+	+	+		+
lmo2511	lmo2511		+	+			+	+		+
lmo2522	lmo2522		-				+			+/-
lmo2527	lmo2527						+			+
lmo2568	lmo2568	+					+			+
lmo2570	lmo2570	-	+	+	+	+	+	+	+	+
lmo2571	lmo2571	-	+		+	+	+	+	+	+
lmo2572	lmo2572	-	+		+	+	+	+	+	+
lmo2573	lmo2573	-	+		+	+	+	+	+	+
lmo2602	lmo2602	-	+	+	+	+	+	+	+	+
lmo2603	lmo2603	-	+		+	+	+	+	+	+
lmo2670	lmo2670	-	+		+	+	+		+	+
lmo2671	lmo2671	-	+		+	+	+	+	+	+
lmo2672	lmo2672	-			+	+	+	+	+	+
lmo2673	lmo2673	-	+	+	+	+	+	+	+	+
lmo2674	lmo2674	-	+		+	+	+	+	+	+
lmo2686	lmo2686						+			+

Table 4.5 (continued)

Gene name	Gene symbol	sigmastatin	Raengpradub et al. 2008	Kazmierczak et al. 2003	Ollinger et al. 2009	Oliver et al. 2010	Toledo-Arana et al. 2009	Hain et al. 2008	σ^B dependent promoter[a]	σ^B OVERALL[b]
lmo2695	lmo2695	-	+	+			+	+		+
lmo2696	lmo2696	-	+				+	+		+
lmo2697	lmo2697	-					+	+		+
lmo2707	lmo2707						+			+
lmo2724	lmo2724	-	+		+	+	+	+	+	+
lmo2733	lmo2733	-				+			+	+
lmo2739	lmo2739	-	+							+
lmo2748	lmo2748	-	+		+	+	+	+	+	+
lmo2785	kat		+					-		+/-

[a] σ^B -dependent promoter was determined by HMM in Oliver et al., 2009.

[b] σ^B –dependence over all studies: Hain et al., 2008, Kazmierczak et al., 2003, Oliver et al., 2009, Ollinger et al., 2009, Raengpradub et al., 2008, Toledo-Arana et al., 2009.

[c] lmo0263 was identified as inhibited by sigmastatin, however, the percent identity for the oligonulceotide probe to sequence match was 80, the cut off for an acceptable probe is ≥90%. Therefore this gene was not considered further.

"+" indicates positively regulated in other studies and "-" indicates negatively regulated in other studies. "+/-" indicates positively and negatively regulated in other studies

regulated by σ^H and not co-regulated by σ^B) was not significantly enriched as a result of sigmastatin treatment (FDR q=0.472). Furthermore, GSEA of the σ^L regulon showed that it was not significantly enriched as a result of treatment with sigmastatin (FDR q=0.836). GSEA was used to determine the biological role category distribution of genes that were differentially affected by sigmastatin. Gene sets representing biological functions including Cellular Processes: Adaptations to Atypical Conditions and Energy Metabolism (other) were enriched amongst sigmastatin-downregulated genes (FDR q=0.060 and q=0.201, respectively). The Cellular Processes: Pathogenesis gene set was also overrepresented amongst sigmastatin-downregulated genes, although just short of significant (FDR q=0.251). Conversely, biological functions such as Cellular Processes: Chemotaxis and Motility, Protein Fate: Protein Folding and Stabilization, and Amino Acid Biosynthesis: Histidine Family were enriched among sigmastatin-upregulated genes (FDR q<0.0001, q=0.008, q=0.031, respectively). GSEA was also performed on the subset of genes that were downregulated by sigmastatin but not σ^B regulated, and it was determined that no role category or regulator, amongst those tested, was enriched in this group of genes (FDR q>0.25).

Genes that showed significantly reduced transcript levels in sigmastatin treated cells include the virulence and *in vivo* viability-associated genes *inlD*, *bilEAB, bsh*, *hfq*, *clpC*, *opuC*, and *gadA* as well as known virulence genes *inlA*, *inlB*. In fact, the regulon of PrfA, the pleiotropic virulence gene regulator, was significantly enriched among the data set, as three genes *inlA, inlB, plcA,* of its small regulon were enriched among sigmastatin downregulated genes (FDR q=0.095). Both *inlA* and *inlB* were significantly downregulated by sigmastatin, however, *plcA,* encoding phospholipase C, was downregulated (adj. p<0.05), but with a fold change of 1.57 it did not meet our cut off criteria of ≥ 2 fold differential transcription. Interestingly, nineteen genes of the Group III PrfA-regulated genes co-controlled by σ^B are both upregulated in the mouse

spleen (14) and inhibited by sigmastatin. This data set includes three genes differentially regulated in the host and identified as potential virulence factors (14), such as lmo1601 (general stress protein), lmo1602 (unknown protein), and lmo2157 (SepA, a metalloprotease in *S. epidermis* (53) and upregulated in *L. monocytogenes* during intracellular infection (16)). A fourth gene inhibited by sigmastatin, also identified as a potential virulence factor by Camejo et al., 2009 (14), is lmo0915, a component of a phosphotransferase system whose regulation has yet to be attributed to any factor. Additionally, sigmastatin also downregulated lmo0937, a gene upregulated in the mouse spleen at 48 hr post-infection and found in the Group III PrfA regulated genes not thought to be controlled by σ^B (14).

Operons identified by Raengpradub et al., 2008 (77) as σ^B –regulated were also significantly differentially transcribed after treatment with sigmastatin, these include *inlAB* (mediates entry into non-professional phagocytes(17)), *opuCABCD* (involved in compatible solute transport), and the 2 gene operon, lmo1699 and lmo1700 (involved in methyl accepting chemotaxis) (Figure 4.5). The autoregulated *sigB* operon consisting of lmo0893-0896 (*rsbV, rsbW, sigB, rsbX*) was downregulated, which likely contributed to further downregulation of the entire regulon. Of note, regulators of σ^B, *rsbX* and upstream gene *rsbU* were recently found to be up regulated during infection in a mouse spleen (14).

Several additional σ^B-dependent genes downregulated by sigmastatin were previously shown to be upregulated during intracellular infection (16) including lmo0232 (*clpC*), lmo0445 (transcriptional regulator), lmo0783 (part of an operon encoding manose phosphotransferase system components, each gene of which is downregulated by sigmastatin) and lmo2672 (also similar to a transcriptional regulator). Furthermore, sigmastatin inhibited cell wall-associated genes, which are

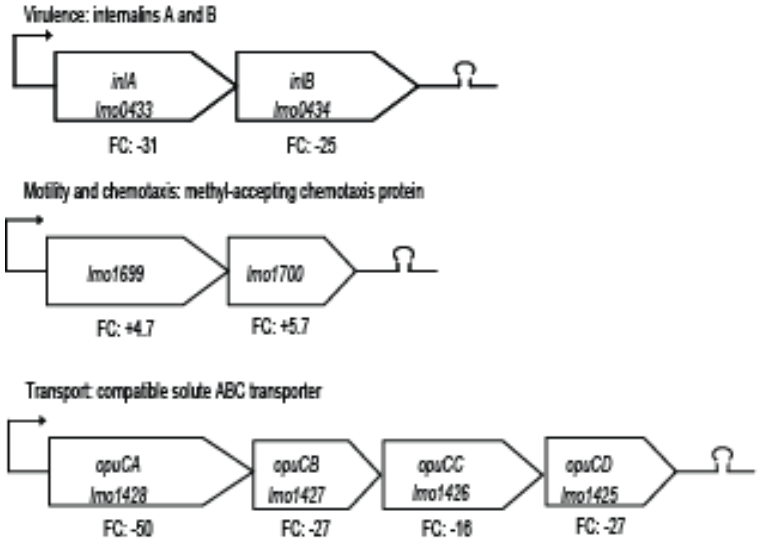


Figure 4.5: σ^B-dependent operons affected by sigmastatin. According to transcriptional profiling of cells treated with sigmastatin, various genes in operons were significantly differentially expressed, including downregulated operons comprised of virulence genes and genes encoding compatible solute transporters. Expression of genes in a motility and chemotaxis operon was upregulated as a result of treatment. As our compound mimics a σ^B null status in the cell, genes that are positively regulated by σ^B will be downregulated by sigmastatin.

upregulated under intracellular conditions (16) and in the murine intestine (98), including *inlA, inlD,* lmo0610, lmo0880, lmo2085 (all of which contain LPXTG sorting motif for cell-wall anchoring) and *inlB* (with a GW domain for mediating binding to host ligands (60)).

Three genes important to glycerol utilization and also required for intracellular listerial growth (43), which were downregulated by sigmastatin, are also regulated by σ^B either positively or negatively under various conditions. Utilization of glycerol as a carbon source in intracellular environments (16) is required for intracellular survival (43). Two of these glycerol utilization genes, lmo1538 (glycerol kinase) and lmo1539 (glycerol uptake facilitator) are downregulated by sigmastatin and are negatively regulated by σ^B in stationary phase and salt stress conditions (77). Interestingly, however, both genes were upregulated by σ^B in the intestine (98) and during intracellular replication (16). The third gene downregulated by sigmastatin, lmo1293, (*glpD*) a glycerol-3-phosphate dehydrogenase, was positively regulated by σ^B in salt (77), intracellularly (43) and in the gastrointestinal tract (98) but downregulated by σ^B in stationary phase (77). The σ^B regulon clearly differs depending on the condition and needs of the bacterial cell (45). In addition to our data, this is further supported by Camejo et al., who found that 40 σ^B dependent genes downregulated in stationary phase (35), were activated *in vivo* in the mouse spleen (14). Furthermore, transcriptional profiling of *L. monocytogenes* in the murine intestine indicates three genes (lmo0642, lmo1251, and lmo1930) exhibited higher expression in the intestine in a WT strain but not Δ*sigB* strain (98). These three genes were also downregulated by sigmastatin. It is likely that several of the genes inhibited by sigmastatin and not previously attributed to σ^B regulation under *in vitro* conditions, are σ^B-dependent but only under very specific conditions, such as those in the mouse intestine. To support this, Camejo et al. demonstrated that expression of genes *in vitro* is indeed lower than

expression of genes *in vivo* (14). Most importantly, however, it is clear that sigmastatin downregulates many genes that enable the bacterium to cause infection.

A smaller proportion of genes upregulated by sigmastatin, as compared to genes downregulated by sigmastatin, are σ^B-dependent

Of the 32 genes upregulated by sigmastatin, only 7 genes were negatively regulated by σ^B in both EGD-e and 10403S strains (35, 77). The σ^B-dependent genes with known functions included an ABC transporter (lmo2114), D-alanine-activating enzyme (*dltA*), post-translocation chaperone (*prsA*), methyl-accepting chemotaxis protein (lmo1699-1700), and NADP glutamate dehydrogenase (lmo0560). Though two genes, lmo2568 (of unknown function) and lmo1637 (similar to membrane protein), were upregulated by sigmastatin, they were also positively regulated by σ^B in the intestine (98) and during various growth phases (35), respectively. Among the non σ^B-dependent genes upregulated by sigmastatin, some were involved in ABC transport, motility and cell-division, but most had unknown functions (Table 4.4).

As previously mentioned, genes ascribed to the motility and chemotaxis role category, were enriched among sigmastatin-upregulated genes according to GSEA. The large flagellar biosynthesis and motility operon (lmo0673-0718) contains 13 recognized σ^B dependent genes (77). Interspersed among these, are six sigmastatin-upregulated genes, which have not been described previously as σ^B-dependent, including lmo0678, lmo0679, lmo0680, lmo0681, lmo0685, lmo0686. Many additional motility genes in this operon were enriched among sigmastatin-upregulated genes (FDR $q<0.0001$), although they were not significantly and differentially expressed. Because of the high number of motility genes affected by sigmastatin, GSEA was performed on the regulons of known chemotaxis and motility related regulators DegU, MogR and CodY. This analysis also showed that the DegU operon

(as defined by Williams et al., 2005 (103)) was enriched in our gene set, among upregulated genes (FDR q<0.0001). Although MogR, the transcriptional repressor of flagellum genes (34, 89), was shown to be σ^B-dependent (98), its regulon (89) was not significantly enriched among our microarray dataset (FDR q=0.257). CodY, the transcriptional repressor of motility and chemotaxis in *B. subtilis* (64), also negatively regulates flagellar components in *L. monocytogenes* (5). Therefore, the genes in the CodY regulon were examined and were found to be significantly enriched among upregulated genes as a result of treatment with sigmastatin (FDR q<0.0001).

We surmise that several new genes, which were significantly and differentially expressed resulting from treatment with sigmastatin, may be σ^B dependent if they are part of an operon containing at least one σ^B dependent gene also affected by sigmastatin. For example, previously only lmo0974 or *dltA* (the first gene in the operon important for modifying lipotechoic and wall techioc acid) was shown to be negatively regulated by σ^B. However, in addition to *dltA*, other genes in this operon (lmo0973 *(dltB)* and lmo0971 *(dltD)*) were also significantly upregulated as a result of treatment of sigmastatin. Therefore, it is possible that *dltB* and *dltD* are also σ^B dependent, yet have not been discovered as such because of less consistent negative regulation.

Noticeably more σ^B-dependent genes were inhibited by sigmastatin than were induced, therefore, σ^B-dependent genes from multiple conditions previously tested (35, 45, 72, 77, 98) were compared to identify trends in σ^B regulation. Only a very small core group of σ^B negatively regulated genes (14 of 264 total) were recurrent in multiple assays (i.e. genes that were identified as negatively regulated by σ^B in two or more assessments of σ^B dependence). Conversely, there was a large core group of σ^B positively regulated genes (137 of 282) (i.e. genes that were identified as positively regulated by σ^B in two or more assessments of σ^B dependence) (Table 4.5). Therefore,

it seems that the accuracy of predicting a "regulon" of σ^B repressed genes based on one assay condition is lower than for positively regulated genes, as seen by increased variability among σ^B repressed genes from different assays. This variability is likely a consequence of the differences introduced at each level of regulation by other transcription factors that overlap in regulation of so-called σ^B-repressed genes (Table 4.5). It is evident however, that sigmastatin truly targets the core σ^B regulon, as it inhibited transcription of 125 of the 137 genes that were positively regulated by σ^B in two or more assays.

Sigmastatin reduces *L. monocytogenes* invasion of human enterocytes

To quantify the effect of sigmastatin on *L. monocytogenes* invasion in Caco-2 human enterocytes, the invasion capacity of exponential phase *L. monocytogenes* cells was assessed after exposure to a low concentration of NaCl (0.3M) known to induce σ^B-activity and treatment with or without sigmastatin. Treatment with sigmastatin, at both 64μM and 8μM, significantly reduced *L. monocytogenes* invasion capacity by approximately 1.4 and 1.5 logs, respectively, producing a 25 and 32 fold reduction in invasion capacity as compared to WT invasion (Figure 4.6; $p<0.05$).]This provides clear phenotypic evidence that sigmastatin hinders virulence functions regulated by σ^B, which are critical to the establishment of orally acquired listeriosis (30).

Inhibition of σ^B in *B. subtilis* indicates specificity across genera

In order to determine if sigmastatin could inhibit σ^B activity in the closely related Gram-positive microbe *B. subtilis*, a β-galactosidase enzymatic assay monitoring a σ^B-dependent *ctc-lacZ* reporter fusion in *B. subtilis* was utilized. This assay showed that treatment with sigmastatin at 64μM significantly inhibited σ^B-

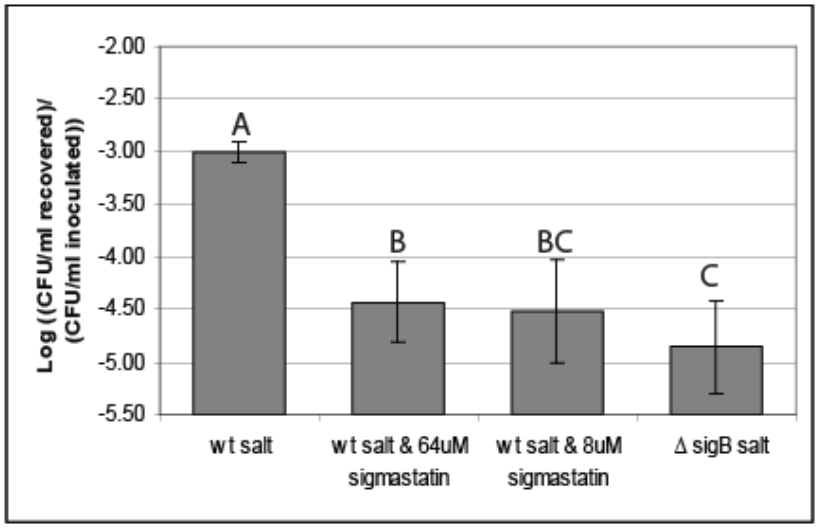


Figure 4.6: Invasion assay. Bar graph of invasion efficiency of *L. monocytogenes* in the human intestinal epithelial cell line Caco-2. Strains and corresponding treatments are indicated on the x-axis. These include wildtype treated with 0.3M NaCl (wt salt), wildtype treated with 0.3M NaCl & 64μM sigmastatin (wt salt & 64μM sigmastatin), wildtype treated with 0.3M NaCl & 8μM sigmastatin (wt salt & 8μM sigmastatin), and the isogenic sigB null strain treated with 0.3M NaCl (Δ*sigB* salt). Invasion efficiency was calculated as the number of bacteria recovered relative to the number of bacteria used for inoculation (i.e., Log ([CFU/ml recovered] /[CFU/ml inoculated]). Data represent four biological replicates, each performed in triplicate. Bars with different letters indicate strain/treatments that differed significantly ($p<0.05$; GLM Tukey). These experiments demonstrate the utility of the identified compound at inhibiting attachment and invasion of human enterocytes.

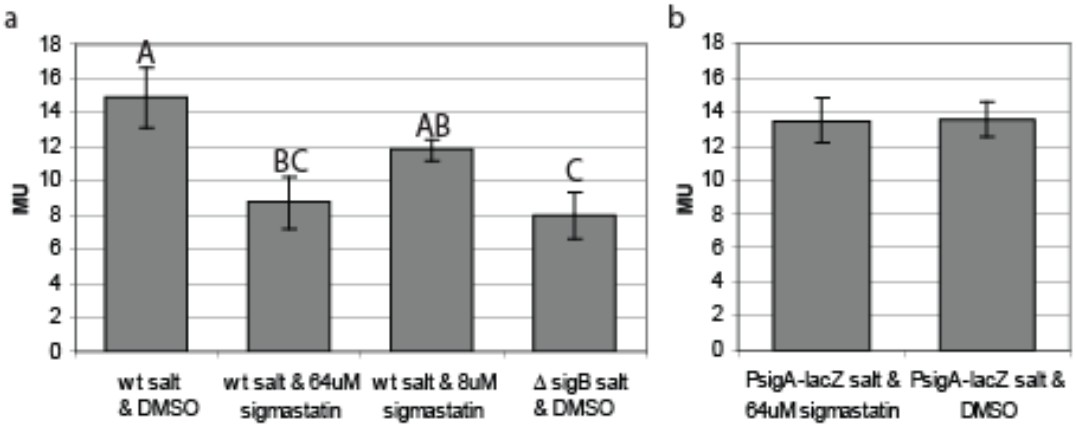


Figure 4.7: *B. subtilis* β-galactosidase assay. Bar graph of β-galactosidase activity in Miller Units (MU) from *B. subtilis* strains with **(a)** σ^B-dependent P*ctc-lacZ* reporter fusions exposed to various conditions. These include wildtype treated with 0.3M NaCl and DMSO (wt salt), wildtype treated with 0.3M NaCl & 64μM sigmastatin (wt salt & 64μM sigmastatin), wildtype treated with 0.3M NaCl & 8μM sigmastatin (wt salt & 8μM sigmastatin), and the isogenic sigB null strain treated with 0.3M NaCl (Δ*sigB* salt) and DMSO. **(b)** shows σ^A-dependent P*rsbRSTU-lacZ* fusion treated with either 0.3M NaCl & 64uM sigmastatin (PsigA-lacZ salt & 64μM sigmastatin) or 0.3M NaCl & DMSO (PsigA-lacZ salt & DMSO). Data represent at least three biological replicates. Bars with different letters indicate strain/treatments that differed significantly ($p<0.05$; GLM Tukey). Sigmastatin (64μM) inhibits σ^B activity in *B. subtilis*, producing *lacZ* levels similar to those in a *B. subtilis* Δ*sigB* strain. Some inhibition also occurs at 8μM concentration of sigmastatin. Sigmastatin treatment does not affect σ^A activity. This data shows the identification identified an effective and specific inhibitor of σ^B-dependent gene expression, which is effective in different Gram-positive bacteria (i.e., the genera *Listeria* and *Bacillus*).

dependent *ctc lacZ* enzyme activity ($p<0.05$; GLM Tukey) almost 2 fold, to levels equivalent to a Δ*sigB* strain (Figure 4.7a ; $p>0.05$). Treatment with 8μM sigmastatin also reduced σ^B-dependent enzyme activity, however, not significantly from WT salt treated cells ($p>0.05$; GLM Tukey). Notably, a σ^A-dependent *lacZ* fusion (104) showed no difference in β-galactosidase activity when treated with 64μM sigmastatin or DMSO (Figure 4.7 b), further pointing to the specificity of sigmastatin for inhibiting σ^B.

DISCUSSION

Using a high-throughput screen of 57,000 small molecules, 41 candidate compounds were identified as potential inhibitors of the *L. monocytogenes* σ^B alternative sigma factor; subsequent screens produced one compound that specifically interfered with σ^B activity. Sigmastatin selectively inhibited σ^B-mediated transcription as shown by qRT-PCR of σ^B-dependent genes and whole-genome microarray analysis of cells treated with the compound relative to untreated cells. This compound also prevented *L. monocytogenes* invasion into human intestinal epithelial cells and inhibited σ^B-directed activity in the Gram-positive bacterium *B. subtilis*. Overall our data show (i) novel small molecules can inhibit the σ^B regulon with high specificity and yield transcriptional profiles similar to a genetic null mutation of the *sigB* gene and (ii) one such compound can be used to prevent expression of *L. monocytogenes* virulence factors important to disease etiology, also inhibiting σ^B activity across genera. Thus, these results provide further evidence that chemical genetics is a valuable approach for identifying potential novel anti-infective therapeutics via targeting transcription factors.

The identified small molecule inhibits σ^B regulon with high specificity and yields transcriptional profiles similar to a genetic null mutation.

A conserved protein is a valuable target against which novel anti-infectives can be developed because of their potential to provide broad-spectrum anti-virulence drugs. In fact, screens have identified a class of inhibitors effective against the conserved type 3 secretion system (TTS) in pathogens *Yersinia spp.*, *Salmonella spp., Shigella flexneri, P. aeruginosa*, *Escherichia coli*, and *Chlamydia spp*. (3, 39, 44, 66, 68, 69, 105). Similarly, from a collection of 150,000 compounds, a highly effective inhibitor of the virulence-associated membrane histidine sensor kinase QseC in enterohemorrhagic *E. coli* (78) was also found to inhibit QseC homologs in other pathogens (i.e., *S.* Typhimurium, *Francisella tularensis*). In addition, Lieberman et al. used a small molecule screen to identify neuroleptic drugs that have potential as therapeutics for various intracellular bacterial pathogens, including *L. monocytogenes* (55, 56). These studies indicate that compounds identified as interfering with virulence-associated characteristics in one pathogen may be effective in a broad range of bacterial pathogens.

Limited work, however, has been performed to identify novel inhibitors of transcription factors for therapeutic use. In a eukaryotic system, Koehler et al., 2003 (52) used a small molecule microarray and transcriptional profiling to identify a small-molecule inhibitor of the Hap3p subunit of Hap 2/3/4/5p yeast transcription factor, whose regulation of mitochondrial function is relevant as a model system for identifying inhibitors of human diseases such as diabetes and cancer. The identified compound produced a transcriptional profile equivalent to a chemical genetic knockdown of Hap2/3/4/5p. In prokaryotes, there have been some successes in identifying small molecules that inhibit members of the AraC family of transcriptional regulators. This family of regulators, like σ^B, contributes to the transcription of

multiple stress response (54) and virulence factors (6, 27), providing a target whose activity is broad in scope, rather than a target whose discrete virulence-associated gene product is unique to a single organism (9). In fact, Hung et al. identified a small molecule inhibitor of *V. cholerae* virulence transcriptional regulator ToxT (an AraC/XylS transcriptional regulator) that inhibited the transcription of critical virulence components: cholera toxin and toxin co-regulated pilus (40). Small molecules have also been used to target other proteins from the AraC family of bacterial transcription factors, including MAR proteins MarA, SoxS and Rob in *E. coli* (9) and LcrF in *Yersinia spp* (49), preventing regulator-DNA binding. These compounds were successful at inhibiting virulence *in vitro* and *in vivo*, suggesting that targeting a pathogen at the transcriptional level is very effective method for inhibition. Another screen identified compounds that inhibit binding of β^1 of core RNAP to σ^{70} in *E. coli* (31). To our knowledge, ours is the first evidence for the identification of a small-molecule inhibitor of a bacterial alternative sigma factor that targets a large majority of its regulon, producing a transcription profile similar to that of a sigma factor null strain thus mimicking the loss of functional σ^B in a cell.

The small molecule identified here, sigmastatin, targets σ^B at an IC_{50} of 3.5uM. The observed activity levels in this low micromolar range are promising because the minimal bacteriocidal concentrations of gentamycin, ampicillin, and streptomycin (against *L. monocytogenes*) are in the range of 2 - 46 μM (63) and the ToxT-inhibiting virstatin (40) showed an MIC between 3 and 40 μM (depending on the target strain). According to whole-genome microarray analysis, 64μM sigmastatin treatment produced inhibition of 55% (156/282) of all genes shown to be positively regulated by σ^B under at least one condition (of 7 assessed) and inhibition of >91% (125/137) of genes positively regulated by σ^B under two or more assay conditions (35, 45, 71, 72, 77, 98). Observation of multiple assays indicates that while 264 genes were shown to

be repressed by σ^B under at least one condition in multiple assays (35, 45, 72, 77, 98), only 14 were differentially expressed under two or more conditions. Interestingly, sigmatatin upregulated only 7 of these 264 σ^B repressed genes, none of which are among the 14 σ^B-dependent genes identified under two or more conditions, suggesting limited utility in targeting negatively regulated genes. The results of treatment with this compound may highlight the more central role of σ^B as a positive regulator with a strong core of positively regulated genes and an indirect role as a negative regulator with very limited set of genes directed under multiple conditions.

Toledo-Arana et al. (98) identified 172 genes in *L. monocytogenes* EGDe, which were up regulated by σ^B in the mouse intestinal lumen. Sigmastatin significantly downregulated 126 of these genes and significantly upregulated 1 of these genes. Of these genes, 17 were σ^B-dependent specifically in the intestinal infection (98). Not only is σ^B is critical to *L. monocytogenes* adaptation and virulence in the intestine (98) and throughout the gastrointestinal tract in its entirety (29), but it also regulates genes involved in intracellular survival and proliferation (16). The effectiveness of this compound at producing a σ^B null status in the cell, inhibiting genes which are important during intestinal infection and preparation for systemic infection as well as adaptation for the intracellular environment, strongly suggests that this compound holds promise as an excellent therapeutic or prophylactic for the treatment of listeriosis.

Small molecules targeting σ^B can be used to probe stress response and regulatory networks in *L. monocytogenes*

To also ascertain whether sigmastatin activity was specific to σ^B, the effect of our anti-infective compound on other alternative sigma factors, including σ^H and σ^L, was assessed. σ^H is important to growth in minimal and alkaline media (80) and

previous microarray analysis showed that much of the σ^H regulon overlaps with that of σ^B. Therefore, the effect of sigmastatin on the σ^H regulon was evaluated. It was determined that the majority of the σ^H-regulated genes that were affected by the compound were also regulated by σ^B and that the σ^H-only regulon (the set of genes in which σ^B co-regulated genes were removed) was not enriched. σ^L, also known as RpoN, contributes to carbohydrate metabolism and antimicrobial resistance (2, 81) and is associated with σ^B in so doing (74). The σ^L regulon, however, was not enriched in our microarray dataset. Moreover, this compound exhibited specificity for this alternative sigma factor in that it not only inhibited *L. monocytogenes* σ^B, but it also inhibited σ^B activity (but had no effect on σ^A activity) in the Gram-positive model organism *B. subtilis*, which is closely related to other low G+C content pathogens. These assessments support the notion that σ^B is a preferential alternative sigma factor target.

Multiple lines of evidence demonstrate a central network between σ^B and PrfA (14, 72), which coordinates *in vivo* expression of genes required for the infectious process (14). The direct regulatory control σ^B has over PrfA via the $P2_{prfA}$ promoter is well established (67, 79, 85), however, it is increasingly clear that there are additional layers of indirect σ^B regulation. As suggested by Ollinger et al., σ^B may act as a posttranscriptional switch that downregulates excessive PrfA activity (72). Those data indicated that multiple virulence genes were differentially expressed in the presence of PrfA* depending on the presence or absence of σ^B. Specifically, σ^B moderated the PrfA regulon in a PrfA* background helping to mediate host-cell damages effects of PrfA-dependent virulence genes (72). In our dataset, the PrfA regulon was significantly enriched among sigmastatin-downregulated genes. The fact that two PrfA-dependent genes, lmo0937 and *plcA*, with no known association with σ^B regulation, were both downregulated by an otherwise σ^B-specific inhibitor

(sigmastatin), suggests there are additional and complex layers of regulation and fine tuning that occur between PrfA and σ^B. However, it is evident that the interplay between these regulators is fundamental in order to co-regulate these subsets of virulence genes to survive and promote infection.

Mounting evidence points to a fundamental role for σ^B in chemotaxis and motility. Specifically, several genes in a large operon of flagellar structural components were identified to be negatively regulated by σ^B (77). Additionally, *sigB* null mutants exhibit increased swarming on agar (77, 98). We surmise that the complex relationship of σ^B to motility could be explained by direct or indirect co-regulation with one or more of the multiple regulators (DegU, MogR and CodY) involved in motility and chemotaxis, allowing for fine-tuning of *L. monocytogenes* response to varied environments. As a result of sigmastatin treatment, several genes important to motility and chemotaxis were upregulated. Specifically, 6 genes on the flagellum biosynthesis operon (lmo0673-0718) were significantly and differentially upregulated. Although they were not among the 13 previously identified σ^B-dependent genes found in this operon (77), they were interspersed among them on the operon. The majority of this flagellar operon is regulated by DegU, a positive activator of flagellum biosynthesis (51), including those 6 sigmastatin upregulated genes (103). Furthermore, 2 genes upregulated by sigmastatin, which comprise the methyl-accepting chemotaxis operon, lmo1699 and lmo1700, are negatively regulated by σ^B (77) and are also regulated by DegU (103). In fact, the DegU and CodY regulons, but not MogR, were significantly enriched among upregulated genes, suggesting an overlap with the negative regulatory function that σ^B has on motility (77, 98). Furthermore, while *Listeria* is known to downregulate flagellar genes during infection (14) to evade the immune system, increased expression of flagellar components can induce potent proinflammatory affects via TLR5-mediated immunogenicity (100).

Therefore, the ability of sigmastatin to upregulate flagellar components validates its potential as an anti-listeriosis drug.

These data regarding the expression of various regulons after treatment with a target-specific compound, such as sigmastatin, support the assertion of complex networks among transcription factors in *L. monocytogenes* (18, 38).

Small molecules targeting transcriptional regulators, including alternative sigma factors, show promise as therapeutic and environmental control agents

Chemical genetics entails the use of small molecules to probe and/or alter biological targets, allowing for a better understanding of certain processes and for identification of novel therapeutics. Identifying compounds that are active against biological targets such as disease-causing mechanisms of pathogenesis in microorganisms has proven to be successful approach for developing new classes of "antibiotics". Specifically, previous work using high-throughput screens to identify small-molecule inhibitors of virulence in *V. cholerae* were successful, allowing for the identification of the small molecule that disrupted protein-protein interactions of transcription factor ToxT, resulting in the prevention and treatment of cholera post-infection. In the high-throughput assay, we found that of the genes inhibited by sigmastatin, 75% were previously reported as σ^B dependent (35, 45, 72, 77, 98). This shows genome wide evidence for inhibition by a highly selective small molecule capable of modulating transcriptional regulation of genes critical to stress response and virulence in the Gram-positive pathogen *L. monocytogenes*.

The alternative sigma factor σ^B is important for responding to stimuli from specific environments including those triggered by transit through the host gastrointestinal tract (4, 29). σ^B modulates gene expression, including expression of stress response and virulence factors, and is therefore important to establishing an

infection in the mammalian host. In addition to σ^B's role in transcription of virulence and *in vivo* viability associated genes, there is burgeoning evidence that σ^B contributes to infection in animal models. In *B. anthracis*, Fouet et al. (28), showed that a deletion mutant of *sigB* was less virulent than the isogenic parental strain. Specifically, there was a one log-unit lower LD_{50} in a *B. anthracis sigB* mutant as compared to the parent strain. The authors suggest that *sigB* may contribute to virulence by allowing *B. anthracis* to persist in the bloodstream of the mammalian host during septicemia, the final stage of anthrax (28). In *Staphylococcus aureus*, σ^B controls a *sarA* promoter and SarA activates *agr*, which in turn encodes a protein that regulates virulence. According to Jonsson et al (42), a *S. aureus* strain, which was defective in σ^B activity because of an impaired posttranslational activator of σ^B (*rsbU)*, showed increased arthritogenicity and sepsis compared to a strain with a repaired *rsbU*. Similar to *B. anthracis*, Jonsson et al. suggested that either σ^B itself or regulation of it by RsbU promotes *S. aureus* survival in the bloodstream, preventing clearance and allowing establishment of infection (42). Furthermore, Lorenz et al. also showed that functional loss of σ^B results in a decrease of *S. aureus* virulence in central venous catheter-related diseases manifested by significantly reduced multiorgan infection caused by σ^B deficient strains (59). In *L. monocytogenes*, Garner et al. showed that as compared to a wildtype strain, a *sigB* null strain of *L. monocytogenes* shows reduced infection in a guinea pig model via an intragastric route (30). Because of its role in virulence and viability of multiple human pathogens in the host, σ^B represents a suitable target for inhibition by novel small molecules.

In our phenotypic and transcriptional profiling experiments, σ^B-dependent virulence genes, such as *inlAB*, *bsh*, *bilE, clpC* and *hfq* were significantly downregulated as a result of treatment with sigmastatin. While each of these genes has been shown to contribute to virulence individually (14, 19, 23, 57, 82, 91), a

compound that can inhibit transcription of all of these genes provides an increased advantage over a compound that targets only one virulence factor (9). Furthermore, stress response and virulence-associated genes *opuC* (92) and *gadA* (20), which are important to survival during passage through the host, were also significantly downregulated by the compound. The ability of this compound to target a wide array of genes required for virulence and *in vivo* viability suggests it has great promise as a therapeutic, as the targeted genes directly contribute to pathogenesis in an animal model.

The Caco-2 human intestinal epithelial cell line provides insight into the interaction between the intracellular pathogen *L. monocytogenes* and intestinal epithelial cells and correlates well to the animal model (30). σ^B is essential for attachment and infection of enterocytes, as demonstrated in Caco-2 human intestinal epithelial cells (30, 48) and is also a requirement for invasion and establishment of infection in the guinea pig model of listeriosis. We used this *in vitro* system to determine the effect sigmastatin has on the ability of *L. monocytogenes* to invade human enterocytes. Sigmastatin severely impedes *L. monocytogenes* attachment and invasion of human intestinal epithelial cells, likely because of the drastically reduced expression of σ^B-directed virulence genes *inlA* and *inlB*. In fact, sigmastatin inhibited σ^B activity to such a degree that it reduced *L. monocytogenes* invasion capacity to that of a Δ*sigB* strain. Most notably, sigmastatin worked rapidly; affecting σ^B directed transcription in less than 10 minutes and subsequent translation in less than 30 minutes of treatment (according to qRT-PCR and invasion assays). This model provides strong phenotypic substantiation that this inhibitory small molecule is able to hinder the virulence functions of σ^B, which are critical to the establishment of orally acquired listeriosis.

Emerging evidence supports that transcription factors in microorganisms are

also promising targets for anti-virulence inhibitors (9, 40, 49, 87). These conserved proteins control multiple genes important to virulence, regulating mechanisms of pathogenesis across multiple microorganisms. This work demonstrates that the regulator critical for *L. monocytogenes* gene expression during infection and stress survival is an excellent target for broad range novel therapeutics. Indeed, from our screen of 57,000 compounds we found that sigmastatin inhibition was specific across genera, substantiating the assertion that such a compound can be used to target homologues of this protein regulator in Gram-positive pathogens. This type of chemical modulator of virulence and *in vivo* viability, which impairs bacterial invasion and persistence in the host via inhibition of genes also increases the microbe's susceptibility to mammalian host defenses. Such a compound can render the organism innocuous and easily cleared by the immune system. This is particularly beneficial for immunocompromised hosts, for whom listeriosis causes the highest morbidity and mortality (25-45%) (32).

Targeting an alternative sigma factor for the development of anti-virulence therapeutics may be beneficial for other diseases for which emerging drug-resistance is thwarting treatment. In fact, this approach might also be applied to alternative sigma factor σ^F (closely related to σ^B) (25, 73) in *Mycobacterium tuberculosis*, which regulates virulence-associated genes important to pathogenesis (90, 93) and antimicrobial resistance (62). This work also establishes a foundation for developing small molecules that can be used to interfere with the ability to survive environmental stress conditions, proving beneficial for controlling transmission and reservoirs of pathogens that persist in the environment. For example, because certain families of alternative sigma factors are conserved, it is possible to use an inhibitor similar to the one discovered in this work, to control sporeformers. Not only are σ^B and σ^F important for many Gram-positive pathogens' survival in the host, they are also important for

enabling survival outside the host prior to infection (99). In fact, a *sigB* deletion strain of *B. cereus*, a foodborne pathogen and close relative of *B. anthracis*, exhibits delayed onset of sporulation and subsequently, less efficient germination (99). Furthermore, development of compounds targeting σ^F, which is important to sporulation in *B. anthracis* (24), may provide a form of environmental control of anthrax. The application of a compound that produces a more susceptible cell is ideal for sensitizing the cell to control agents and other preventative measures. As our data demonstrates, targeting alternative sigma factors is a worthwhile approach to consider for future drug development or environmental control agents for inhibition of microbial transmission.

By utilizing chemical-genetics strategies, identification of anti-virulence agents active against *L. monocytogenes* transcriptional regulators, such as alternative sigma factors, could produce human chemotherapeutics active against a number of similar Gram-positive pathogens. Such strategies would potentially help avoid the misuse of classical antibiotics, preserving their efficacy for situations warranting their application. This approach affords us the opportunity to develop novel agents which abrogate or reduce *L. monocytogenes* pathogenicity and possibly other Gram-positive clinically relevant pathogens, which will help to mitigate the burdens currently beleaguering public health and safety. This research provides a better understanding of the benefits derived from employing chemical genetics in concert with microbiology for drug development for human pathogens. By extrapolating what we learn from one organism and harnessing this knowledge, we can rationally develop drugs intended to target the very factors which are essential to microbial survival in the host and pathogenicity in a number of similar disease-causing microorganisms.

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CHAPTER 5

CONCLUSIONS

Listeria monocytogenes has the highest fatality rate among foodborne pathogens and disproportionately affects infants, the elderly and immunocompromised individuals. The loss of life and financial burdens caused by *L. monocytogenes* warrant investigation into new methods for control and alleviation of this public health threat. Increasing interest in employing chemical genetics to answer questions in the realms of both eukaryotic and prokaryotic biology has permitted a better understanding of biological functioning (e.g. pathways and proteins) (12). Chemical genetics has also lent itself to the identification of valuable compounds with potential for treating human diseases, such as those caused by harmful viruses, parasites, and bacteria (4-6, 8, 11). In particular, this approach has been successfully used to inhibit transcription factors from the AraC family of regulators which contribute to virulence in several bacterial pathogens (1, 2).

Building upon this foundation, we set out to identify new bacterial targets in *L. monocytogenes* for anti-infective development. First, our research aimed to determine the contributions of select transcriptional regulators to virulence and to antimicrobial resistance to select peptides. Results from the research supported that σ^B promotes invasion, PrfA is critical to cell-to-cell growth and showed that CtsR, in addition to PrfA and σ^B, are important to virulence in a guinea pig model of listeriosis. Moreover, σ^B and σ^L both contribute to antimicrobial peptide resistance, coordinating response to both SdpC and nisin. Therefore, we chose the alternative sigma factor σ^B as our target of choice for anti-infective development because of its role in regulating stress response and antimicrobial resistance, promoting *in vivo* viability and contributing to virulence in *L. monocytogenes*. In order to discover novel small molecules capable of

attenuating human listeriosis via inhibition of σ^B, we used a high-throughput format to screen ~57,000 small molecules to identify selective inhibitors of σ^B. The resulting promising compounds were reassessed using a secondary cell-based HTS format as well as qRT-PCR; one select compound was then comprehensively validated through a variety of methods such as transcriptional profiling, phenotypic assessments, and mammalian tissue culture infection models. We identified a potential anti-virulence agent, sigmastatin, with high specificity for σ^B in both *Listeria* and *Bacillus*, producing a chemically induced σ^B null status equivalent to a genetic knockout of σ^B in the cell. This novel agent inhibited invasion thus reducing *L. monocytogenes* pathogenicity pointing to the possibility of application against other Gram-positive clinically relevant pathogens.

We anticipate that this work will further the development of novel therapeutic agents that are detrimental to the pathogenic potential of prokaryotes but entirely benign to eukaryotes. In using the approach described in the work presented here, the ultimate goal is to help reduce public health and safety issues caused by the foodborne pathogen *L. monocytogenes*, while avoiding the misuse of classical antibiotics and preserving them for warranted situations. For the future of this work, the next steps to pursue would involve forming a complete picture of sigmastatin's mode of action. In order to do this: (i) numerous derivatives of this compound will need to be tested in order to determine the most important sub-structures on the molecule that contribute to optimal inhibition and (ii) sigmastatin target-identification will need to be performed. As data from our small-molecule microarray suggested the possibility that σ^B and sigmastatin bind, we hope to elucidate how sigmastatin is inhibiting σ^B activity via identification of true targets. Previous work has shown that small molecules can inhibit protein-protein interactions (9) or protein-DNA interactions (3), thus, we surmise that sigmastatin may act in a number of ways. For instance, it may prevent

effective core RNAP- σ^B association and/or subsequent promoter binding or it may act upstream on regulators of σ^B (Rsb). We suggest using a general target identification method such as SILAC (stable isotope labeling with amino acids in cell culture) (7) or a more specific method such as SPR (surface plasmon resonance). SILAC, which is used in quantitative proteomics, involves a combination of isotope labeling, affinity chromatography and mass spectrometry to identify any potential protein binders to a bead-conjugated small molecule (SM). This method entails the use of one lysate population labeled with a light isotope and exposed to both a bead-affixed SM and a soluble SM (that acts as competitor bait reducing the number of target proteins that bind the SM-bead) and one lysate population labeled with heavy isotope exposed only to the SM-bead. The resulting heavy to light peptide fragment ratio allows for identification of true protein "interactors" using mass spectrometry (7). We might also use SPR to immobilize a variety of potential targets to determine affinity and specificity of protein-ligands pairs or even to determine the effects of a small molecule ligand on a protein complexed with other molecules, such as DNA. Alternatively, a genetic approach can be used to identify the protein target of sigmastatin. This would involve screening for bacteria exhibiting a resistance phenotype, such as mutant colonies able to deconjugate bile salts on selective agar containing inhibitory levels of sigmastatin. Subsequently, the mutant colonies would be assessed to determine a genetic explanation for resistance using total genome sequencing approaches, such as Solexa (Illumina) or 454 sequencing, pinpointing mutations in the target that elicited resistance (10). Ultimately, this information may allow for the realization of a highly effective listeriosis treatment option.

Identification of sigmastatin and a subsequent understanding of its mode of action serve as a stepping stone for the identification of similar compounds targeted against other sigma factors of interest. We anticipate that this may provide an avenue

for the generation of compounds aimed at an expansive array of applications (beyond anti-virulence agents), such as the identification of environmental control agents or simply for improving our current understanding of gene regulation and regulatory networks.

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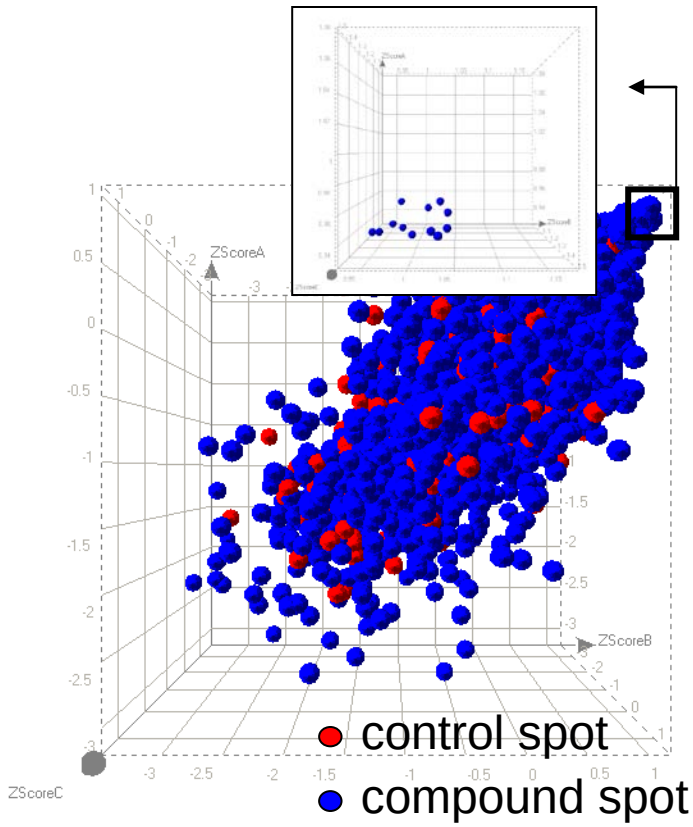
Appendix Table AT.1: Secondary Screening candidates

Virtual ID	Vender ID	Decision	Result of σ^B inhibition
SPBio_000086	Spectrum01500167	Not available commercially	Possible growth inhibitor
SPBio_000596	Spectrum00210477	Effective in secondary, Not available commercially at the time, actinonin	Might work
SPBio_001858	Spectrum00200034	Effective in secondary, Not available commercially at the time, atranorin	Might work
SPBio_002673	Prestwick_000747	Not pursued after secondary, antibiotic	Possible growth inhibitor
Ald1.1-H_000308		Would need resynthesis	Might work
ACon1_001486	NP-005144	Not available	Possible growth inhibitor
ChemDiv3_002815	4151-0291	Not pursued after secondary	Possible growth inhibitor
ChemDiv3_002999	**4237-0075**[a]	Pursued after secondary	Not effective
ChemDiv3_005866	6145-0438	Not pursued after secondary	Possible growth inhibitor
ChemDiv3_005911	6079-1959	Not pursued after secondary	Unclear
ChemDiv3_006137	6228-2502	Not pursued after secondary, aryl hydrazone	Might work
ChemDiv3_007160	8012-2663	Not pursued after secondary	Unclear
ChemDiv3_007374	**8009-2163** [a]	Pursued after secondary, minimal cytotoxicity	Works-used derivative T0513
ChemDiv3_010387	C614-5726	Not pursued after secondary, aryl hydrazone	Might work
ChemDiv3_010413	C660-0131	Not pursued after secondary, cytotoxic	Might work
Maybridge4_001879	JFD00174	Not pursued after secondary	Unclear
Maybridge4_001886	JFD02846	Not pursued after secondary	Unclear
Maybridge4_001932	**JFD03061** [a]	Pursued after secondary	Works- needs optimization
Maybridge4_001966	JFD02331	Not pursued after secondary	Possible growth inhibitor
Maybridge4_001967	JFD00263	Not pursued after secondary	Possible growth inhibitor
Maybridge4_002150	KM06170	Not pursued after secondary, aryl hydrazone	Might work
Maybridge4_002415	KM04727	Not pursued after secondary, aryl hydrazone	Might work
Maybridge4_003857	S13598	Pursued as growth inhibitor, not effective as sigB or growth inhibitor	Not effective
Maybridge4_004192	SEW02081	Not pursued after secondary	Might work
Maybridge4_004329	SP01461	Not pursued after secondary	Possible growth inhibitor
Maybridge4_004330	SPB02585	Not pursued after secondary	Might work
Maybridge4_004364	SPB01534	Not pursued after secondary	Might work
Maybridge4_004503	SP01411	Not pursued after secondary	Unclear
Maybridge4_004548	SPB02493	Not pursued after secondary, aryl hydrazone	Might work
Maybridge4_004591	SPB06794	Not pursued after secondary	Might work
Maybridge4_004694	SPB06723	Not pursued after secondary, aryl hydrazone	Might work
Enamine_001246	T0504-0705	Not pursued after secondary, problems with dilution	Unclear
Enamine_001250	T0500-0388	Not pursued after secondary, problems with dilution	Unclear

Appendix Table AT.1 (Continued)

Enamine_001604	T0505-1745	Not pursued after secondary, aryl hydrazone, problems with dilution, structure similar to 4237-0075	Unclear
TimTec1_003523	ST047887	Not pursued after secondary	Possible growth inhibitor
TimTec1_003545	ST050178	Not pursued after secondary, cytotoxic	Might work-ytotoxic
TimTec1_003817	ST050863	Not pursued after secondary	Possible growth inhibitor
TimTec1_003987	ST057360	Not pursued after secondary	Unclear
TimTec1_005049	ST212074	Not pursued after secondary	Might work
TimTec1_005093	ST211458	Not pursued after secondary	Unclear
TimTec1_007542	ST5024987	Not pursued after secondary	Unclear

[a] Compounds or derivatives of compounds in bold were assessed further using qRT-PCR, etc.



Appendix Figure AF.1: Scatterplot of small-molecule microarray screen. Three-dimensional scatterplot of Z-scores calculated from normalized fluorescence intensity resulting from interaction between σ^B and printed small-molecule ligands. Arrays were tested in triplicate and bound His-tagged σ^B was detected using Alexa Fluor 647 labeled anti-His antibody. Red dots represent DMSO controls; blue dots are small molecules ligands tested.